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## ANNOUNCEMENT

The Central Society for Clinical Research and The C. V. Mosby Company have entered into an agreement which provides that *THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE* will be published as the official publication of the Central Society for Clinical Research. Beginning with Volume 32, this issue, the Central Society for Clinical Research will assume editorial direction of the *JOURNAL*.

The Board of Editors will consider for publication manuscripts which record original contributions in the broad fields of clinical investigation and clinical pathology. A "Methods" section will be retained. The works of *all authors* are invited; society affiliations will not prejudice or influence editorial decisions. The current practice of providing prompt publication of articles accepted by the Board of Editors will be continued.

Many of the papers which will appear in the first three issues were approved by the former editorial board.



# METABOLIC FUNCTION OF PTEROYLGLUTAMIC ACID AND ITS HEXAGLUTAMYL CONJUGATE

## I. HEMATOLOGIC AND URINARY EXCRETION STUDIES ON PATIENTS WITH MACROCYTIC ANEMIA

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THE properties of folic acid as a hematopoietic factor differentiate it clearly from both the dietary extrinsic factor and the antipernicious anemia principle contained in refined liver extracts.<sup>1</sup> Since folic acid occurs in foods almost wholly in conjugated form,<sup>2</sup> it is pertinent to compare the hematopoietic responses of suitable patients with macrocytic anemia following the administration, in sequence, of the vitamin in the bound and free states. Furthermore, an estimate may be obtained of the ability of such patients to release the vitamin from its conjugate by determinations of the urinary excretion of the free material after the oral administration of the conjugate. Because the terminology applied by different authors to folic acid and related compounds is variable and confusing, the free vitamin, folic acid, henceforth will be designated by its chemical name, pteroylglutamic acid, and the conjugate used in these studies will be referred to as pteroylhexaglutamylglutamic acid (hexaglutamyl conjugate).<sup>3</sup> Another conjugate of pteroylglutamic acid has been isolated by Stokstad and co-workers<sup>4</sup> and termed the fermentation *Lactobacillus casei* factor or pteroyltriglutamic acid.

In a previous communication<sup>5</sup> we reported that a naturally occurring conjugated form of folic acid was not as effective as the free vitamin in producing hematopoietic responses in two patients with pernicious anemia in relapse and in one patient with macrocytic anemia following subtotal gastrectomy. Observations leading to a similar conclusion have been described by Welch and associates.<sup>6</sup> At this time we shall present additional data pertaining to the utilization by patients with pernicious anemia and related macrocytic anemias of the hexaglutamyl conjugate as evidenced by their hematopoietic responses and the urinary excretion of the free vitamin after administration of the conjugate. These studies were initiated by the consideration that in pernicious anemia there is an apparent deficiency of the vitamin, pteroylglutamic acid, which cannot, in most cases, be explained by dietary inadequacy.

The specific disturbances of erythropoiesis (namely, megaloblastic proliferation and defective maturation) which characterize many of the macro-

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cytic anemias have suggested that a common functional derangement was operative in their production. This concept was greatly strengthened by the work of Castle and associates<sup>7</sup> who demonstrated convincingly the essential role played by diet and by gastric secretion in the maintenance of normal human erythrocyte formation; it received additional support from the observation that a single form of therapy was generally effective in macrocytic anemias of widely varying clinical background. As a consequence, most studies bearing on the etiology of clinically encountered or experimentally produced macrocytic anemia were concerned with attempts to relate the condition under observation to deficiency of a gastric (intrinsic) or a dietary (extrinsic) factor or to some disturbance in the absorption or utilization of the product of their interaction.<sup>8</sup> Although this theory appeared to be generally consistent with factual observations, its universal application was made difficult by the accumulation of evidence that some cases of macrocytic anemia, particularly those associated with nutritional deficiencies and pregnancy, failed to respond adequately to parenteral administration of concentrated liver principle but showed reversion to normal erythropoiesis when cruder preparations of liver were given either by oral or parenteral routes or when autolyzed yeast was administered by mouth. The existence of a hematopoietic substance in crude liver extracts and yeast, other than the antipernicious factor present in purified liver extracts, was first postulated by Wills and Evans.<sup>9</sup> They concluded from controlled observations that lack of this substance was responsible for their cases of tropical macrocytic anemia as well as for an experimentally produced macrocytic anemia of monkeys<sup>10</sup> and suggested that a similar deficiency in sprue might result from failure of absorption. They identified the substance tentatively as a part of the vitamin B complex and pointed out that it differed from dietary extrinsic factor if the hematopoietic activity of the latter, in accordance with the theory of Strauss and Castle,<sup>8</sup> was limited to a role in the formation of the liver principle.

Day and associates<sup>11</sup> recognized independently in monkeys a macrocytic anemia prevented or cured by administration of yeast or liver extracts. They named the curative factor in these materials vitamin M.<sup>12</sup> More recently, Day and co-workers<sup>13</sup> and also Wilson and associates<sup>14</sup> showed that the deficiency syndrome in monkeys could be successfully treated with purified *L. casei* factor (pteroylglutamic acid). Hogan and Parrott<sup>15</sup> were the first to describe an anemia resulting from a dietary deficiency in chicks. They designated the hematopoietic agent vitamin B<sub>c</sub>. Vitamin B<sub>c</sub> was subsequently isolated<sup>16</sup> and now has been shown to be identical with pteroylglutamic acid.<sup>3</sup> A similar nutritional deficiency characterized by leucopenia and anemia has been produced in rats largely as a result of the work of Daft, Sebrell, and their associates.<sup>17</sup>

An analysis of the literature dealing with the relative effectiveness of refined liver extracts and cruder preparations of liver, whole liver, or autolyzed yeast in the treatment of human macrocytic anemias was recently published by Watson and Castle.<sup>18</sup> These workers reported four cases of nutri-

tional macrocytic anemia. In three of the patients there was no response to parenterally administered liver extract in dosages effective in the treatment of addisonian pernicious anemia, but there were good hematologic and clinical results when a liver extract was given by mouth. In a fourth patient a response was obtained only when the parenterally administered dose of liver extract was increased ten times. They recognized the presence in crude liver extracts and autolyzed yeast of the hematopoietic substance, described by Wills and Evans,<sup>9</sup> and suggested for it the designation of "Wills' factor."

The observations of Wintrobe<sup>19</sup> on the effects of brewers' yeast on erythropoiesis in pernicious anemia support the view that patients with this disease may be able to utilize some substance other than the "liver principle." Earlier investigators, to whom Wintrobe refers, demonstrated the effectiveness of yeast, usually given in autolyzed form in the treatment of some cases of pernicious anemia, but Wintrobe first seriously questioned the validity of attributing the hematopoietic effect of yeast exclusively to its extrinsic factor content. His data reveal the variability of responses by patients with pernicious anemia to the administration of yeast.

With the demonstration by several groups of investigators of the effectiveness of pteroylglutamic acid in the treatment of pernicious anemia and other macrocytic anemias,<sup>20-24</sup> it appeared probable that the activity of crude liver and yeast in the studies referred to previously was due to their content of pteroylglutamic acid in free or conjugated form.

Earlier observations on the therapeutic effectiveness of pteroylglutamic acid in human anemic and leucopenic states were handicapped by lack of availability of the vitamin in pure form or in relatively concentrated amounts. Thus, the inconclusive or negative results obtained by Sharp and associates,<sup>25, 26</sup> Castle and co-workers,<sup>27</sup> Vilter and Spies,<sup>28</sup> and Moore and associates<sup>22</sup> may be attributed to administration of too small amounts of effective material, as suggested by Sharp, or to the use of material in a form not readily utilizable by persons with pernicious anemia.

#### CLINICAL MATERIAL AND EXPERIMENTAL PROCEDURE

In the course of this study data were secured from nine patients with addisonian pernicious anemia in relapse and four with other macrocytic (megaloblastic) anemias; of these, two followed gastrectomy, one was probably on a nutritional basis with some features of the nontropical sprue syndrome, and one was associated with chronic diffuse liver disease. Data were also obtained on the urinary excretion of pteroylglutamic acid by three patients with pernicious anemia in liver extract-induced remission. All patients with pernicious anemia fulfilled the diagnostic requirements of macrocytic anemia with characteristic morphologic changes in the peripheral blood, megaloblastic marrow hyperplasia, achlorhydria persisting after parenteral administration of histamine, and ultimate hematopoietic response to treatment with either pteroylglutamic acid or the intramuscular injection of refined liver extract.

Hemoglobin and hematocrit determinations and cell counts were made at regular intervals, using oxalated venous blood, and reticulocyte percentages were obtained daily throughout the period of observation on all patients in relapse. The urinary excretion of pteroylglutamic acid was determined by microbiologic assay using *L. casei*.<sup>2</sup> No evidence was obtained of the excretion of the conjugated vitamin as such. While studies were being carried out, the subjects were given a low protein, meat-free diet of adequate caloric value.

The procedure employed in performing the clinical studies, with some individual modifications, was as follows. Urine collected and suitably preserved during the first twenty-four hours of study was used for the quantitative determination of urobilinogen.<sup>29</sup> Subsequently, for at least two days, the urinary output of pteroylglutamic acid was measured while the patient received the standard diet without medication. A yeast concentrate supplying known amounts of hexaglutamyl conjugate was then given daily for varying periods up to fourteen days, followed by the administration of synthetic pteroylglutamic acid<sup>30, 31</sup> in a dosage equivalent to that of the vitamin present in the conjugate. In one case a period of conjugate administration was followed by one in which the same yeast concentrate was given after incubation with a specific enzyme capable of liberating the vitamin from its bound form. The properties of the hexaglutamyl conjugate are described in the accompanying communication in which are reported the results of urinary excretion studies on normal individuals.<sup>32</sup>

#### CASE REPORTS AND EXPERIMENTAL RESULTS

##### *I. Hematopoietic Responses of Patients With Macrocytic Anemia Following the Administration of Conjugated and Free Pteroylglutamic Acid*

CASE 1.—H. W., a 63-year-old white man, fulfilled all diagnostic criteria of pernicious anemia and had had the disease for approximately four years. Although the anemia was severe the patient appeared to be in fairly good nutritional state, was ambulatory, and had no complicating conditions. He complained of numbness and tingling of the extremities but had no disturbance of gait and no objective signs of central nervous system degeneration. During the preliminary period of observation and diet standardization it became advisable to give the patient a transfusion because of extreme weakness and progression of anemia. Accordingly, 500 c.c. of whole blood were administered and subsequently he was given daily a yeast concentrate containing the equivalent of 4 mg. of pteroylglutamic acid as hexaglutamyl conjugate. After he had received this material for ten days with no hematopoietic response, treatment was changed to synthetic pteroylglutamic acid in a daily oral dose of 4 milligrams. The hematologic data obtained on this patient are shown in Table I.

CASE 2.—N. J., a 56-year-old white man, was first admitted to the Simpson Memorial Institute in 1936, when the diagnosis of pernicious anemia was made and he responded in the expected manner to the parenteral administration of liver extract. During the intervening ten years the patient had received liver therapy very irregularly and none had been taken for several months prior to the present admission. The diet appeared to be adequate particularly with respect to protein from animal sources. There were no subjective or objective manifestations of nervous system involvement. The patient was not given a transfusion, and after a preliminary period without treatment he was given the hexaglutamyl conjugate in a dosage equivalent to 4 mg. of the free vitamin. A small increase in reticulocytes occurred which, since this patient at the same time showed a

TABLE I. HEMATOLOGIC DATA ON CASE 1, H. W.

DATE	TREATMENT	DAY	R.B.C. × 10 <sup>6</sup>	HEMO- GLOBIN (GM.)	HEMA- TOCRIT (%)	RETICU- LOCYTES (%)	W.B.C.
2/28/46	None	1	1.1	4.9	13.5	0.6	3,250
3/ 1/46	None	2	--	--	--	0.2	----
3/ 2/46	None	3	--	--	--	0.6	----
3/ 3/46	None	4	--	--	--	0.7	----
3/ 4/46	None	5	--	--	--	0.1	----
3/ 5/46	Blood, 500 c.c.	6	1.0	3.6	12.0	0.2	1,700
3/ 6/46		7 (0)	1.3	4.8	15.5	0.4	1,700
3/ 7/46		1	--	--	--	0.2	----
3/ 8/46		2	--	--	--	0.1	----
3/ 9/46		3	--	--	--	0.1	----
3/10/46	Conjugate equivalent to 4 mg. PGA	4	--	--	--	0.1	----
3/11/46		5	--	--	--	0.1	----
3/12/46		6	--	--	--	0.1	----
3/13/46		7	1.2	4.5	14.0	0.6	----
3/14/46		8	--	--	--	0.7	----
3/15/46		9	--	--	--	0.8	----
3/16/46		10 (0)	1.2	4.2	13.5	0.1	2,960
3/17/46		1	--	--	--	0.3	----
3/18/46		2	--	--	--	0.6	----
3/19/46		3	--	--	--	0.2	----
3/20/46	PGA, 4 mg.	4	--	--	--	0.5	----
3/21/46	PGA, 4 mg.	5	--	--	--	1.4	----
3/22/46	PGA, 4 mg.	6	--	--	--	2.1	----
3/23/46	PGA, 4 mg.	7	--	--	--	2.8	----
3/24/46	PGA, 4 mg.	8	--	--	--	3.2	----
3/25/46	PGA, 4 mg.	9	--	--	--	7.1	----
3/26/46	PGA, 4 mg.	10	1.3	4.2	16.0	9.3	3,050
3/27/46	PGA, 4 mg.	11	--	--	--	7.1	----
3/28/46	PGA, 4 mg.	12	--	--	--	8.2	----
3/29/46	PGA, 4 mg.	13	--	--	--	5.3	----
3/30/46	PGA, 4 mg.	14	1.6	5.0	19.0	1.6	4,600

TABLE II. HEMATOLOGIC DATA ON CASE 2, N. J.

DATE	TREATMENT	DAY	R.B.C. × 10 <sup>6</sup>	HEMO- GLOBIN (GM.)	HEMA- TOCRIT (%)	RETICU- LOCYTES (%)	W.B.C.
3/22/46	None	1	2.0	7.9	24.0	1.0	5,500
3/23/46	None	2	--	--	--	0.6	----
3/24/46	None	3	--	--	--	0.7	----
3/25/46		4 (0)	1.8	7.3	23.0	0.8	3,850
3/26/46		1	--	--	--	0.6	----
3/27/46		2	--	--	--	0.6	----
3/28/46		3	--	--	--	1.4	----
3/29/46		4	--	--	--	0.6	----
3/30/46	Conjugate equivalent to 4 mg. PGA	5	--	--	--	2.0	----
3/31/46		6	--	--	--	1.7	----
4/ 1/46		7	--	--	--	3.4	----
4/ 2/46		8	--	--	--	1.4	----
4/ 3/46		9	--	--	--	1.4	----
4/ 4/46		10	1.9	7.4	24.0	4.1	3,250
4/ 5/46		11	--	--	--	3.7	----
4/ 6/46		12 (0)	1.9	7.3	24.0	3.7	3,300
4/ 7/46		1	--	--	--	3.6	----
4/ 8/46		2	--	--	--	4.0	----
4/ 9/46	Conjugate equivalent to 4 mg. PGA incubated with con- jugase	3	--	--	--	2.6	----
4/10/46		4	--	--	--	4.1	----
4/11/46		5	--	--	--	2.7	----
4/12/46		6	--	--	--	4.9	----
4/13/46		7	--	--	--	6.9	----
4/14/46		8	--	--	--	4.2	----
4/15/46		9	2.8	9.2	32.5	4.8	7,400



slight rise in urinary excretion level of pteroylglutamic acid, probably indicates some ability to liberate and utilize the conjugated form of the vitamin. During the second part of the experiment the patient received the same yeast concentrate in identical dosage but previously incubated with a specific conjugase derived from hog kidney. This enzyme has been shown to liberate pteroylglutamic acid from the hexaglutamyl conjugate.<sup>2</sup> The quantity of hog kidney tissue employed and the method of preparation of the conjugase-containing extract were such as to render it extremely unlikely that any response could be attributed to the presence of antipermeicous substance in hog kidney. A suboptimal but definite reticulocyte rise occurred during administration of this material, together with a substantial increase in erythrocytes and hemoglobin and decided clinical improvement. Hematologic data obtained on this patient are presented in Table II.

CASE 3.—H. P., a 65 year old white man, underwent subtotal gastrectomy for adenocarcinoma of the stomach three years before the present admission. Approximately seven-eighths of the stomach was removed. There were no evidences of metastases at the time

TABLE III HEMATOLOGIC DATA ON CASE 3, H. P.

DATE	TREATMENT	DAY	R.B.C. × 10 <sup>6</sup>	HEMO- GLOBIN (GM.)	HEMA- TOCRIT (%)	RETICU- LOCYTES (%)	W.B.C.
2/21/46	None	1	1.8	8.1	25.5	1.2	4,250
2/22/46	None	2	—	—	—	—	—
2/23/46	None	3	—	—	—	1.4	—
2/24/46	None	4	—	—	—	0.6	—
2/25/46	None	5	—	—	—	0.5	—
2/26/46	Conjugate equivalent to 2.3 mg. PGA	6 (0)	2.2	7.1	26.5	0.4	4,600
2/27/46		1	—	—	—	0.3	—
2/28/46		2	—	—	—	0.2	—
3/ 1/46		3	—	—	—	0.4	—
3/ 2/46		4	—	—	—	0.6	—
3/ 3/46	PGA, 2.3 mg.	5	—	—	—	0.4	—
3/ 4/46		6	—	—	—	0.6	—
3/ 5/46		7	—	—	—	1.6	—
3/ 6/46		8 (0)	1.8	7.5	25.5	1.6	5,400
3/ 7/46		1	—	—	—	1.8	—
3/ 8/46	PGA, 2.3 mg.	2	—	—	—	1.4	—
3/ 9/46	PGA, 2.3 mg.	3	—	—	—	2.1	—
3/10/46	PGA, 2.3 mg.	4	—	—	—	2.6	—
3/11/46	PGA, 2.3 mg.	5	—	—	—	2.8	—
3/12/46	PGA, 2.3 mg.	6	—	—	—	1.4	—
3/13/46	PGA, 2.3 mg.	7	—	—	—	1.5	—
3/14/46	PGA, 2.3 mg.	8	—	—	—	1.7	—
3/15/46	PGA, 2.3 mg.	9	—	—	—	1.0	—
3/16/46	PGA, 5.0 mg.	10 (0)	2.5	8.5	30.5	1.2	5,800
3/17/46	PGA, 5.0 mg.	1	—	—	—	1.1	—
3/18/46	PGA, 5.0 mg.	2	—	—	—	0.9	—
3/19/46	PGA, 5.0 mg.	3	—	—	—	1.2	—
3/20/46	PGA, 5.0 mg.	4	—	—	—	1.1	—
3/21/46	PGA, 5.0 mg.	5	—	—	—	1.0	—
3/22/46	PGA, 5.0 mg.	6	—	—	—	1.0	—
3/23/46	FeSO <sub>4</sub> , 2.0 Gm. added	7	2.8	7.5	27.5	0.4	5,950
3/24/46	FeSO <sub>4</sub> , 2.0 Gm. added	8	—	—	—	0.3	—
3/25/46	FeSO <sub>4</sub> , 2.0 Gm. added	9	—	—	—	1.2	—
3/26/46	FeSO <sub>4</sub> , 2.0 Gm. added	10	—	—	—	0.4	—
3/27/46	FeSO <sub>4</sub> , 2.0 Gm. added	11	—	7.7	29.5	0.6	—
3/28/46	FeSO <sub>4</sub> , 2.0 Gm. added	12	—	—	—	0.4	—
3/29/46	FeSO <sub>4</sub> , 2.0 Gm. added	13	3.1	9.6	35.5	0.6	4,000

of the operation or later. Gradual development of weakness and fatigability occurred about one and one-half years before the current admission. Although the dietary habits were modified by the necessity of taking frequent small meals, largely in order to avoid symptoms of postgastrectomy hypoglycemia, the quality of the food was not restricted and, since the patient had received detailed dietary instructions following the operation, the diet may be assumed to have been somewhat better than the average for a person of his age and economic status. The patient's symptoms were attributable to the anemia and physical examination was negative except for pallor and moderate underweight. The urine was negative except for 1 plus protein and the stool examination revealed no occult blood and no evidence of parasitism. This patient received yeast concentrate supplying the hexaglutamyl conjugate in a daily dosage equivalent to 2.3 mg. of the free vitamin for eight days with no hematopoietic response. Subsequently he was given synthetic pteroylglutamic acid in the same dose. There was only a slight increase in reticulocyte percentage but a definite rise in erythrocytes and hemoglobin. Hematologic data are shown in Table III.

CASE 4.—E. B., a 76-year-old white man, fulfilled the diagnostic criteria of pernicious anemia and had had symptoms for approximately two years. The diet had been deficient particularly with respect to meat and vegetables, but the patient had included in his average daily intake six slices of bread, one egg, and one quart of milk. He had lost about 7 pounds during the eight months preceding the admission to the hospital. He had no gastrointestinal complaints other than moderate anorexia. Numbness and tingling of the feet with some difficulty in walking in the dark had been present for three months. The only objective neurologic abnormalities were moderately increased deep reflexes of upper and lower extremities, bilateral, and slight impairment of vibratory sense in the feet. Treatment with yeast concentrate supplying hexaglutamyl conjugate in daily dosage equivalent to 5 mg. of pteroylglutamic acid was carried out for eight days with no hematopoietic response. Since other metabolic studies were performed subsequently on this patient, it was not possible to demonstrate an initial response to administration of the free vitamin alone. However, for three months the patient has received pteroylglutamic acid, 5 mg. daily by mouth, without other medication, and during this period the blood values reached normal levels and remission has been well maintained. Hematologic data obtained on this patient are shown on Table IV.

TABLE IV. HEMATOLOGIC DATA ON CASE 4, E. B.

DATE	TREATMENT	DAY	R.B.C. × 10 <sup>6</sup>	HEMO- GLOBIN (GM.)	HEMA- TOCRIT (%)	RETICU- LOCYTES (%)	W.B.C.
8/ 1/46	None	1	1.5	5.8	17.0	2.5	5,100
8/ 2/46	None	2	—	—	—	1.6	—
8/ 3/46	None	3	—	—	—	2.3	—
8/ 4/46	None	4	—	—	—	1.4	—
8/ 5/46	None	5	—	—	—	1.8	—
8/ 6/46	Conjugate equivalent to 5 mg. PGA	6 (0)	1.4	5.3	16.5	2.0	5,200
8/ 7/46		1	—	—	—	2.4	—
8/ 8/46		2	—	—	—	0.8	—
8/ 9/46		3	—	—	—	4.7	—
8/10/46		4	—	—	—	3.1	—
8/11/46		5	—	—	—	3.4	—
8/12/46		6	—	—	—	2.8	—
8/13/46		7	1.4	5.7	16.5	2.8	5,600

CASE 5.—G. L., a 76-year-old white man, complained chiefly of diarrhea and swelling of the legs. Beginning about four years prior to admission to the hospital the diarrhea at first had been periodic, but for the last five months it had been constant with from three to six watery yellow stools each day. Swelling of the legs had been present and had progressed for more than one year. Weakness, ease of fatigue, and dyspnea on exertion had developed over a period of five years, and during this time the patient noted numbness

and tingling of the lower extremities, ataxia, and a burning sensation in his feet. Periodic soreness of the tongue had been present for about one year. He also complained of burning and itching of the eyes. The diet was grossly inadequate, consisting chiefly of bread and potatoes. The patient prepared his own food and bought one-half pound of hamburger each week. No other meat was taken and the diet contained very little milk, eggs, green vegetables, or fruit. His appetite was poor and the patient's weight had decreased from a maximum of 155 pounds to 117 pounds on admission and 100 pounds after diuretic therapy.

Examination revealed the following signs: severe emaciation; dry, shiny skin with many follicular keratoses and irregular areas of brownish pigmentation; vascularizing keratitis and corneal opacities; fissures at the angles of the mouth; a smooth, magenta tongue with some fiery red areas; and other evidences of riboflavin and nicotinic acid deficiency. There was generalized arteriosclerosis. The blood pressure was 108 systolic and 70 diastolic. The heart findings were not abnormal and the lungs were clear except for rales at the bases. The abdomen was negative. Pronounced pitting edema of the lower extremities was present bilaterally. Knee and ankle jerks were not elicited, vibratory sense was absent below the knees, and sense of motion and position was impaired. There was calf muscle tenderness and light pressure over the toes elicited a sensation of burning.

TABLE V. HEMATOLOGIC DATA ON CASE 5, G. L.

DATE	TREATMENT	DAY	R.B.C. × 10 <sup>6</sup>	HEMO- GLOBIN (gm.)	HEMA- TOCRIT (%)	RETICU- LOCYTES (%)	W.B.C.
8/19/46	None	1	--	--	---	0.5	----
8/20/46	None	2	--	--	---	1.0	----
8/21/46	None	3	--	--	---	0.3	----
8/22/46	Conjugate equivalent to 5 mg. PGA	4 (0)	2.4	9.4	29.5	0.5	5,100
8/23/46		1	--	--	---	0.6	----
8/24/46		2	--	--	---	0.7	----
8/25/46		3	--	--	---	0.9	----
8/26/46		4	--	--	---	1.5	----
8/27/46		5	--	--	---	1.2	----
8/28/46		6	--	--	---	2.6	----
8/29/46		7	2.8	10.9	31.5	2.9	5,100

Macrocytic anemia of moderate degree was present. Sternal marrow aspiration revealed increased and disordered erythropoiesis with numerous megaloblasts. Histamine refractory achlorhydria and absence of pepsinogen were demonstrated. Total serum proteins were 6.5 Gm. per 100 c.c. with albumin globulin ratio of 1.6. All other laboratory examinations including hepatic and renal function tests gave essentially normal results. Roentgen study of the gastrointestinal tract revealed profound small bowel dysfunction. "At no point in the course of the small intestine can one find normal bowel pattern. Irregularity of lumen width is pronounced. 'Puddling' of barium is equally obvious." (Dr. F. J. Hodges.)

This patient received the same yeast concentrate as those discussed previously supplying the hexaglutamyl conjugate in a daily dose equivalent to 5 mg. of pteroylglutamic acid. During an eight day period of conjugate administration little evidence of a reticulocyte response was noted although a significant increase in erythrocytes and hemoglobin occurred (Table V). Moreover, as will be shown later, the patient excreted a relatively large amount of free vitamin during administration of the yeast concentrate. Although this patient obviously suffered with multiple nutritional deficiencies and small bowel disease, it is not possible to exclude Addisonian pernicious anemia in view of the achylia gastrica. However, his failure to improve over a period of three months, with persistence of anemia, diarrhea, eye and tongue changes, and further weight loss, strongly suggests that the pathologic process is quite distinct from that responsible for pernicious anemia. Little or no improvement has followed therapy with pteroylglutamic acid in daily doses of

15 mg. by oral and parenteral routes of administration, large doses of refined and crude liver extracts by intramuscular and intravenous injection, and the intravenous administration of plasma and vitamin B fractions. The nervous system manifestations in this case are not severe and may be entirely attributable to peripheral nerve disease. There is no evidence of active infection to explain the failure of response to specific therapy.

CASE 6—G. W., a 74 year old white man, developed symptoms of anemia six years prior to admission to the hospital. He was treated with injections of liver extract and improved, but treatment became increasingly irregular. Numbness and tingling of the feet had been present for eighteen months and periodic burning of the tongue for six months. Weakness gradually became so severe that he was unable to leave his home. Although his appetite had been poor for three months and he had lost about 10 pounds during this period, the diet did not appear to be qualitatively seriously deficient. The patient was fairly well nourished but extremely pale. The tongue was smooth with reddening along the lateral margins. Patellar reflexes were diminished and vibratory sense was absent over the left foot and ankle, elsewhere over the lower extremities it was greatly decreased. In other respects the physical findings were essentially normal. The results of laboratory studies, including achlorhydria and megaloblastic marrow reaction, were characteristic of pernicious anemia. This man received a yeast concentrate differing from that given to the patients previously discussed in that by *in vitro* test it possessed only one seventh as much conjugase inhibitor activity. It has been shown by Bird and associates<sup>27</sup> that yeast extracts contain a strong inhibitor for pteroylhexaglutamylglutamic acid conjugase and that this inhibitor is gradually removed during fractionation of yeast extract in the prepa-

TABLE VI. HEMATOLOGIC DATA ON CASE 6, G. W.

DATE	TREATMENT	DAY	R. B. C. × 10 <sup>6</sup>	HFMG GLOBIN (GM.)	HFMG TCRIT (%)	RETICULOCYTES (%)	W. B. C.
10/ 7/46	None	1	1.8	7.1	21.0	0.3	4,950
10/ 8/46	None	2	—	—	—	0.4	—
10/ 9/46	None	3	—	—	—	0.4	—
10/10/46	None	4	—	—	—	0.6	—
10/11/46	Conjugate, with low conjugase inhibiting activity, equivalent to 4 mg. PGA	5 (0)	1.8	6.5	22.0	0.2	3,100
10/12/46		1	—	—	—	0.3	—
10/13/46		2	—	—	—	0.2	—
10/14/46		3	—	—	—	0.4	—
10/15/46		4	—	—	—	0.1	—
10/16/46		5	—	—	—	0.2	—
10/17/46		6	—	—	—	0.5	—
10/18/46		7	1.7	6.4	21.0	1.4	4,350
10/19/46		8	—	—	—	2.5	—
10/20/46		9	—	—	—	3.1	—
10/21/46		10	—	—	—	9.1	—
10/22/46		11	1.9	7.3	24.0	11.6	3,550
10/23/46		12	—	—	—	16.2	—
10/24/46		13	—	—	—	9.9	—
10/25/46		14	2.3	9.2	28.0	7.9	6,250
10/26/46		15	—	—	—	3.3	—
10/27/46		16	—	—	—	2.2	—
10/28/46		17	—	—	—	3.5	—
10/29/46		18	—	—	—	2.8	—
10/30/46		19	—	—	—	1.6	—
10/31/46		20	—	—	—	2.8	—
11/ 1/46	PGA, 4 mg	21 (0)	2.4	8.3	29.0	3.4	3,950
11/ 2/46	PGA, 4 mg	1	—	—	—	2.7	—
11/ 3/46	PGA, 4 mg.	2	—	—	—	2.0	—
11/ 4/46	PGA, 4 mg.	3	—	—	—	1.5	—
11/ 5/46	PGA, 4 mg.	4	—	—	—	1.2	—
11/ 6/46	PGA, 4 mg.	5	—	—	—	1.8	—
11/ 7/46	PGA, 4 mg.	6	—	—	—	0.8	—
11/ 8/46	PGA, 4 mg	7	2.8	9.1	32.5	1.0	4,000

ration of pure conjugate. A hematopoietic response was obtained during administration of this yeast concentrate supplying as conjugate the equivalent of 4 mg. of pteroylglutamic acid daily. No second reticulocyte increase followed the oral administration of the same dose of the free vitamin (Table VI).

CASE 7.—T. K., a 69-year-old white woman, fulfilled all diagnostic criteria of pernicious anemia and had first experienced symptoms of anemia five months previously. Numbness and tingling of the feet had been present for about five years with no ataxia or disturbance of gait. The patient was moderately obese and had no evidences of nutritional deficiency. The tongue was reddened and smooth along the lateral margins. Patellar reflexes were diminished and vibratory sensation was impaired over both lower extremities. This patient denied having received any form of antianemia therapy prior to admission, and she was observed in the hospital for fourteen days before treatment was instituted. During this period no hematopoietic response occurred but on the day treatment was started an increase in reticulocyte percentage was observed. The subsequent course of the reticulocyte rise was of a type that strongly indicated a specific response to the material administered, but the high initial value suggested that the marrow at the time of institution of therapy was in a reactive state. She received the same yeast concentrate as that given to G. W. (Case 6) in daily dosage equivalent to 4 mg. of pteroylglutamic acid, and later suggestive but not conclusive evidence was obtained of a second reticulocyte response to treatment with the free vitamin (Table VII).

TABLE VII. HEMATOLOGIC DATA ON CASE 7, T. K.

DATE	TREATMENT	DAY	R.B.C. × 10 <sup>6</sup>	HEMO- GLOBIN (GM.)	HEMA- TOCRIT (%)	RETICU- LOCYTES (%)	W.B.C.
10/11/46	None	1	2.5	8.1	30.0	1.2	5,300
10/12/46	None	2	--	--	--	0.8	----
10/13/46	None	3	--	--	--	1.4	----
10/14/46	None	4	--	--	--	2.4	----
10/15/46	None	5	--	--	--	2.4	----
10/16/46	Conjugate, with low conjugase inhibiting activity, equivalent to 4 mg. PGA	6 (0)	2.5	8.6	26.5	5.0	4,200
10/17/46		1	--	--	--	8.2	----
10/18/46		2	--	--	--	7.3	----
10/19/46		3	--	--	--	5.0	----
10/20/46		4	--	--	--	7.4	----
10/21/46		5	--	--	--	10.5	----
10/22/46		6	--	--	--	13.6	----
10/23/46		7	2.9	10.0	30.5	14.1	5,800
10/24/46	PGA, 4 mg.	8	--	--	--	20.0	----
10/25/46		9 (0)	3.0	10.1	31.5	11.9	6,400
10/26/46		1	--	--	--	10.1	----
10/27/46		2	--	--	--	8.0	----
10/28/46		3	--	--	--	14.6	----
10/29/46		4	--	--	--	10.6	----
10/30/46		5	3.3	10.9	35.0	10.1	6,700

The data obtained from observations of the hematopoietic responses of Cases 1 to 7 indicate that patients with proved pernicious anemia in relapse are unable to utilize in any appreciable degree the hexaglutamyl conjugate of pteroylglutamic acid when the material is supplied orally together with relatively large amounts of a conjugase inhibiting substance. The responses obtained when a conjugate source containing less enzyme inhibitor was administered suggest that the defective utilization of natural forms of pteroylglutamic acid by patients with pernicious anemia is quantitative and probably varies with the individual patient.

## II. Urinary Excretion of Pteroylglutamic Acid During Administration of the Hexaglutamyl Conjugate and the Free Vitamin to Patients With Macrocytic Anemia in Relapse

In this study determinations of the twenty-four-hour urinary excretion of pteroylglutamic acid were performed on the patients previously discussed (Cases 1 to 7) and on six additional patients who could not be tested for hematopoietic responses either because of their relatively high initial erythrocyte values or their unwillingness to submit to protracted courses of therapy. The data secured from the urinary assays on all of the subjects are compiled in Table VIII. Brief abstracts of the six additional cases follow.

CASE 8.—O. B., a 39-year-old white man, had had periodic soreness of the tongue for three years and symptoms of anemia for four months. The clinical and laboratory findings were entirely characteristic of pernicious anemia. The patient had received no previous antianemia therapy. Initial hematologic values were: red cells, 1,500,000 per cubic millimeter; hemoglobin, 6.4 Gm. per 100 c.c.; hematocrit, 19 per cent; mean corpuscular volume, 112 cubic microns; mean corpuscular hemoglobin concentration 33.7 per cent; white cells, 4,750 per cubic millimeter. Clinical and hematologic remission was attained while the patient was receiving pteroylglutamic acid, 5 mg. daily.

CASE 9.—L. P., a 63-year-old white woman, had been treated for pernicious anemia with striking symptomatic improvement five years previously. She had had no treatment for about four years prior to the admission. She presented the characteristic features of pernicious anemia with initial blood values as follows: red cells, 900,000 per cubic millimeter; hemoglobin, 3.6 Gm. per 100 c.c.; hematocrit 11.0 per cent; mean corpuscular volume, 122 cubic microns; mean corpuscular hemoglobin concentration, 32.8 per cent; white cells, 3,050 per cubic millimeter. Because of the severity of the anemia a single blood transfusion was given. The patient has subsequently attained complete clinical and hematologic remission while receiving pteroylglutamic acid, 5 mg. by mouth daily.

CASE 10.—H. B., a 71-year-old white woman, had symptoms of weakness, numbness and tingling of the extremities, and soreness of the tongue of approximately one year's duration. She had macrocytic anemia, megaloblastic marrow reaction, and histamine refractory achlorhydria. An initial hematopoietic response was obtained during oral administration of pteroylglutamic acid, 5 mg. daily, but complete remission was induced by intramuscular injection of refined liver extract, 15 units weekly. The hexaglutamyl conjugate was not given to this patient, and urinary excretion data are limited to observations during the administration of the free vitamin. The initial hematologic values were as follows: red cells, 1,700,000 per cubic millimeter; hemoglobin, 6.4 Gm. per 100 c.c.; hematocrit, 20 per cent; mean corpuscular volume, 118 cubic microns; mean corpuscular hemoglobin concentration, 32 per cent; white cells, 4,800 per cubic millimeter.

CASE 11.—D. R., a 55-year-old white woman, had onset of numbness and tingling and disturbances of gait eight months previously. Soreness of the tongue had been present for several weeks. Examination revealed evidence of moderately advanced posterolateral column degeneration. Quantitative blood cell changes were minimal, but definite variations in red cell morphology were observed including anisocytosis and macrocytosis. Histamine refractory achlorhydria was demonstrated. After excretion studies were completed, decided symptomatic improvement and restoration of blood values to normal followed treatment with refined liver extract by intramuscular injection. The initial hematologic data were as follows: red cells, 4,100,000 per cubic millimeter; hemoglobin, 13.3 Gm. per 100 c.c.; hematocrit, 41 per cent; white cells, 4,850 per cubic millimeter.

TABLE VIII. URINARY EXCRETION OF PTEROYLGLUTAMIC ACID BY PATIENTS

CASE NUMBER		1	2	3	4	5
	DAYS					
Daily excretion of PGA during pretreatment period	1	0.3 $\gamma$	1.8 $\gamma$	1.4 $\gamma$	0.6 $\gamma$	1.7 $\gamma$
	2	0.5 $\gamma$	2.3 $\gamma$	2.0 $\gamma$	0.4 $\gamma$	1.3 $\gamma$
	3				0.6 $\gamma$	
	4				0.6 $\gamma$	
Conjugate daily dose as PGA equivalent		4.0 mg.	4.0 mg.	2.3 mg.	5.0 mg.	5.0 mg.
Daily excretion of PGA during period of conjugate administration	1	1.5 $\gamma$	14.8 $\gamma$	2.3 $\gamma$	4.3 $\gamma$	1,195.0 $\gamma$
	2	1.6 $\gamma$	13.0 $\gamma$	3.1 $\gamma$	5.8 $\gamma$	437.0 $\gamma$
	3				5.7 $\gamma$	612.0 $\gamma$
	4			2.2 $\gamma$	33.5 $\gamma$	672.0 $\gamma$
	5				68.0 $\gamma$	900.0 $\gamma$
	6				72.0 $\gamma$	830.0 $\gamma$
	7					1,340.0 $\gamma$
	8			2.8 $\gamma$		
Average percentage excretion of dose administered		0.028 %	0.30 %	0.04 %	0.337 %	17.1 %
PGA as free vitamin; daily dose		4.0 mg.	4.0 mg.	2.3 mg.	5.0 mg.	5.0 mg.
Daily excretion of PGA during period of administration of free vitamin	1	98.8 $\gamma$	723.0 $\gamma$	45.0 $\gamma$	1,115.0 $\gamma$	592.8 $\gamma$
	2	233.1 $\gamma$	761.0 $\gamma$	80.0 $\gamma$	974.4 $\gamma$	420.0 $\gamma$
	3				1,082.4 $\gamma$	921.3 $\gamma$
	4	261.3 $\gamma$			1,246.4 $\gamma$	980.0 $\gamma$
	5					
	6	784.4 $\gamma$				
	7					
	8	1,130.0 $\gamma$				
Average percentage excretion of dose administered		12.5 %	18.5 %	5.4 %	22.8 %	14.5 %

CASE 12.—T. N., a 56 year old white man, developed macrocytic anemia five years after total gastrectomy for gastric ulcer. The blood cell values rapidly became normal while the patient was receiving pteroylglutamic acid, 5 mg. daily by mouth.

CASE 13.—E. J. B., a 64 year old white woman, had macrocytic anemia of mild degree associated with evidences of chronic diffuse liver disease. For one year she had received refined liver extract, 15 units intramuscularly every one to two weeks, and some improvement in blood values had followed this therapy. Shortly before admission to the hospital the patient had an exacerbation of the hepatic disorder with rapidly developing painless jaundice and enlargement of the liver, associated with evidences of increased hepatic dysfunction obtained by laboratory tests. It was during this period of heightened activity of the disease process that pteroylglutamic acid excretion studies were performed. Subsequently the patient recovered from the acute exacerbation of the illness and at present is much improved.

The excretion of free vitamin during oral administration of the hexaglutamyl conjugate to patients with pernicious anemia in relapse and macrocytic anemia following gastrectomy was quite variable. Nevertheless, as has been shown by the percentage calculations on Table VIII, only a small fraction of the dose was excreted in the urine, whereas a much greater part of the orally administered free vitamin was excreted. Comparison of the data obtained on these subjects with those secured during similar studies on normal persons reveals that the patients with pernicious anemia and postgastrectomy macrocytic anemia have much lower excretion levels of pteroylglutamic acid during conjugate administration.<sup>32</sup> Although a difference in excretion values during administration of the free vitamin to these patients and to the normal subjects was also observed, it was much less than that found when the conjugate was given. Moreover, as

## MACROCYTIC ANEMIA DURING ADMINISTRATION OF CONJUGATED AND FREE VITAMIN

6	7	8	9	10	11	12	13
0.6 γ 1.5 γ	1.3 γ 0.6 γ 0.6 γ 1.6 γ	2.8 γ 0.9 γ 0.9 γ	0.4 γ	0.4 γ 0.3 γ	1.8 γ	2.1 γ 2.2 γ	1.2 γ
4.0 mg.	4.0 mg.	5.0 mg.	4.0 mg.	----	----	4.0 mg.	4.0 mg.
62.0 γ 32.0 γ 85.0 γ 85.2 γ 72.8 γ 44.1 γ 220.0 γ 303.6 γ 2.83 %	1.9 γ 1.6 γ 4.1 γ 8.6 γ 8.6 γ 14.9 γ 15.2 γ 16.8 γ 0.20 %	3.2 γ 2.8 γ 5.5 γ	2.0 γ 1.4 γ 2.3 γ 2.0 γ			4.5 γ 4.2 γ	2.4 γ 2.4 γ
4.0 mg. 339.0 γ 336.0 γ	4.0 mg. 135.2 γ 196.8 γ 234.3 γ 334.6 γ 215.0 γ	5.0 mg. 576.0 γ 1,277.0 γ	----	5.0 mg. 219.6 γ 499.2 γ	5.0 mg. 242.6 γ 501.7 γ	4.0 mg. 486.4 γ	4.0 mg. 9.0 γ
1,876.0 γ							
8.41 %	4.22 %	18.5 %	----	17.3 %	7.4 %	12.13 %	0.2 %

illustrated by Cases 1 and 10, continuous administration of the free vitamin was associated with excretion values increasing to levels equaling those obtained on normal subjects. This was not observed when the hexaglutamyl conjugate was fed continuously in a yeast concentrate possessing strong conjugase inhibiting activity (Cases 3 and 9). Case 4 (E. B.) who also received the conjugate showed a definite increase in excretion of the free vitamin, but the maximum level attained after six days of administration was less than the lowest value observed in the group of normal subjects.

Special consideration must be given to the excretion values obtained in Cases 5, 6 and 7. They will be reviewed together with the pertinent hematologic data in the discussion to follow.

In Case 13, macrocytic anemia associated with liver disease, very low excretion values for pteroylglutamic acid were observed after both conjugate and free vitamin administration.

### *III. The Effect of Liver Extract (Parenteral) on the Urinary Excretion of Pteroylglutamic Acid During Administration of Its Hexaglutamyl Conjugate to Pernicious Anemia Patients*

Refined liver extract was given by intramuscular injection to a patient with pernicious anemia in relapse who had received no previous antianemia therapy (Case 8). The dose employed was 15 U. S. P. units contained in 1 c.c. given on each of three consecutive days. Hexaglutamyl conjugate was taken orally for three days before institution of liver extract therapy and during the period of its administration. No significant change in the excretion levels of pteroyl-



glutamic acid was observed (Table IX). A similar study was carried out on another patient with pernicious anemia (Case 1) after the administration of synthetic pteroylglutamic acid, 10 mg. daily by mouth for seven weeks. During the period of pteroylglutamic acid therapy the erythrocyte count increased from 2,100,000 to 3,300,000 per cubic millimeter. Refined liver extract was injected intramuscularly for six consecutive days in a daily dosage of 15 U. S. P. units except that on the second day of treatment the patient received 45 units. In this case also no change in the excretion of free vitamin was observed during simultaneous administration of hexaglutamyl conjugate and liver extract (Table X).

TABLE IX. EFFECT OF SIMULTANEOUS ADMINISTRATION OF HEXAGLUTAMYL CONJUGATE AND PARENTERAL LIVER EXTRACT ON URINARY EXCRETION OF PTEROYLGLUTAMIC ACID (PGA) BY PATIENT O. B., CASE 8

DATE	TREATMENT	DAY	EXCRETION OF ( $\mu$ G) PGA PER 24 HOURS
9/16/46	None	1	2.3
9/17/46	None	2	0.9
9/18/46	None	3	0.9
9/19/46	None	4	--
9/20/46	Conjugate with high enzyme inhibitor content equivalent to 5 mg. PGA	1	3.2
9/21/46		2	2.8
9/22/46		3	5.5
9/23/46	Same plus refined liver extract 15 units, I. M.	1	5.8
9/24/46		2	--
9/25/46		3	5.7

TABLE X. EFFECT OF SIMULTANEOUS ADMINISTRATION OF HEXAGLUTAMYL CONJUGATE AND PARENTERAL LIVER EXTRACT ON URINARY EXCRETION OF PTEROYLGLUTAMIC ACID (PGA) BY PATIENT H. W., CASE 1

DATE	TREATMENT	DAY	EXCRETION OF ( $\mu$ G) PGA PER 24 HOURS
5/28/46	None	1	--
5/29/46	Conjugate with high enzyme inhibitor content equivalent to 4 mg. PGA	1	33.1
5/30/46		2	
5/31/46	Same plus refined liver extract 15 units, I. M.	1	54.6
6/ 1/46		2	27.2
6/ 2/46		3	14.8
6/ 3/46	15 units, I. M.	4	11.0
6/ 4/46	15 units, I. M.	5	31.9
6/ 5/46	15 units, I. M.	6	16.8

Three patients with pernicious anemia in liver extract-induced remission were studied with respect to their excretion of free vitamin during administration of hexaglutamyl conjugate.

CASE 14.—W. M., a 72-year-old white man, was found to have pernicious anemia thirteen years previously. He has been under continuous observation since then, has received parenteral liver extract regularly, and has never been in relapse.

CASE 15.—N. M., a 64-year-old white woman, was given the diagnosis of pernicious anemia fifteen years before the present studies. Throughout the period she has been treated with parenteral liver extract and the hematopoietic values have been maintained within normal limits.

CASE 16.—A. G., a 66-year-old white man, presented the diagnostic features of pernicious anemia sixteen years previously and for a number of years was treated with desiccated stomach. For the past five years he has received intramuscular injections of liver extract. The patient has always been irregular in following the treatment schedule, and although he has never been in severe relapse, the blood cell values have frequently indicated that therapy was inadequate. When pteroylglutamic acid excretion studies were undertaken the erythrocyte count was 4,100,000 per cubic millimeter, hemoglobin 13.5 Gm. per 100 c.c. and hematocrit 44.5 per cent.

All three of these patients received their initial diagnoses at the Simpson Memorial Institute on the basis of clinical and hematologic features of pernicious anemia, histamine refractory achlorhydria, and subsequent response to antipernicious anemia therapy.

After a preliminary period during which the patients received the standard low protein diet and base line excretion values for pteroylglutamic acid were obtained, they were given the hexaglutamyl conjugate in a daily dose equivalent to 4 mg. of the free vitamin. The source of conjugate was the yeast concentrate possessing strong enzyme inhibiting activity. The last injection of liver extract had been given to these patients about one week previously and none was administered during the excretion studies. The urinary values for pteroylglutamic are presented on Table XI.

It appears to be significant that the excretion levels of pteroylglutamic acid, during the preliminary period when they received the standard diet only, were higher in these patients than in those who were studied during relapse. The base line excretion values of the patients in liver extract-induced remission equal those observed in normal subjects.<sup>32</sup> The excretion of free vitamin during the period of conjugate administration was greater in the patients in remission than in any of the patients with proved pernicious anemia in relapse who received the same yeast concentrate (Table VIII).

TABLE XI. URINARY EXCRETION OF PTEROYLGLUTAMIC ACID (PGA) DURING ORAL ADMINISTRATION OF HEXAGLUTAMYL CONJUGATE AND FREE VITAMIN TO PATIENTS WITH PERNICIOUS ANEMIA IN LIVER EXTRACT-INDUCED REMISSIONS

CASE NUMBER		14	15	16
	DAYS			
Daily excretion of PGA during pretreatment period	1	4.4 $\mu$ g	2.7 $\mu$ g	2.5 $\mu$ g
	2	4.6 $\mu$ g	1.8 $\mu$ g	2.2 $\mu$ g
Conjugate daily dose as PGA equivalent		4.0 mg.	4.0 mg.	4.0 mg.
Daily excretion of PGA during period of administration of conjugate in concentrate with high enzyme inhibitor content	1	147.0 $\mu$ g	54.5 $\mu$ g	34.4 $\mu$ g
	2	268.8 $\mu$ g	167.0 $\mu$ g	40.0 $\mu$ g
	3			71.2 $\mu$ g
	4			96.6 $\mu$ g
	5			51.5 $\mu$ g
	6			173.8 $\mu$ g
	7			60.8 $\mu$ g
Average percentage excretion of dose administered		5.1 %	3.0 %	1.8 %
PGA as "free" vitamin; daily dose		4.0 mg.	4.0 mg.	----
Daily excretion of PGA during period of administration of free vitamin	1	564.0 $\mu$ g	287.5 $\mu$ g	----
	2	800.0 $\mu$ g	215.6 $\mu$ g	----
Average percentage excretion of dose administered		16.9 %	6.2 %	----

## DISCUSSION

It may be presumed that the excretion of pteroylglutamic acid after conjugate administration is dependent upon several factors, aside from renal impairment. These are: (1) the liberation of the vitamin from the conjugate, (2) its absorption from the alimentary tract, (3) the extent of tissue deficiency, and (4) the presence and functional status of tissue enzyme systems required for pteroylglutamic acid utilization.

In pernicious anemia three of these factors may be operative in varying degrees. Liberation of the free vitamin may be adversely affected by defective intestinal conjugase activity aggravated by the presence of enzyme inhibiting substances. The extent of tissue deficiency may influence the retention of absorbed vitamin and so inversely affect its excretion rate. The status of cellular enzyme systems may govern the capacity of the tissues to utilize the vitamin.

The patients in this series who received the hexaglutamyl conjugate together with relatively large amounts of enzyme inhibitor exhibited little or no hematopoietic response. The low excretion levels of pteroylglutamic acid, therefore, may be attributed largely to deficient conversion of the conjugate to the free form. Case 6 (G. W.) who received a conjugate in a preparation with low inhibitor content had moderately high initial and subsequently increasing levels of vitamin excretion. This observation together with the definite but suboptimal hematopoietic response suggests that a significant amount of pteroylglutamic acid was liberated and absorbed but that in spite of tissue need, as shown by the severity of the anemia, there was imperfect utilization of the vitamin. Case 7 (T. K.), at the time of institution of therapy with the same conjugate as that given in Case 6, showed evidences of spontaneous erythrocyte regeneration. The initial reticulocyte percentage was 5.0 and the rise during treatment was rapid, reaching a peak of 20.0 per cent on the eighth day. This value is greater than the expected maximum reticulocyte peak during adequate oral therapy with liver extract or desiccated stomach for a patient with her pretreatment red blood cell count.<sup>34</sup> There can be little doubt that the increase in reticulocytes and total number of circulating red cells, observed in this patient, represents a response to the material administered. The low excretion values for pteroylglutamic acid, as shown on Table VIII, although apparently inconsistent with the observations on other patients in this series, may be attributable to a high degree of tissue deficiency and unusually efficient cellular utilization of the liberated and absorbed vitamin. This concept is supported by the observation in this case of exceptionally low vitamin excretion levels during administration of synthetic pteroylglutamic acid, in the presence of continued hematopoietic response.

Case 5 (G. L.) was discussed earlier with emphasis upon the clinical evidences of multiple nutritional deficiencies refractory to specific therapy. There appear to be adequate grounds for considering this case as one of probable nutritional macrocytic anemia rather than true pernicious anemia. The excretion of unusually high levels of free vitamin during administration of a source of conjugate possessing strong conjugase inhibiting activity is proof of

absorption of the material from the alimentary tract in spite of the small bowel dysfunction and diarrhea. The failure to obtain a sustained hematopoietic response to the subsequent parenteral administration of pteroylglutamic acid and crude and refined liver extracts and the lack of therapeutic effect of large doses of thiamine, riboflavin, and nicotinic acid, administered both orally and parenterally, indicate that in this patient irreversible tissue changes have occurred probably relating to cellular enzyme activity.

These observations suggest that in cases of nutritional macrocytic anemia, even though complicated by extensive disturbances of small bowel function, the liberation and absorption of conjugated pteroylglutamic acid in the presence of conjugase inhibitor are not necessarily impaired as they appear to be in Addisonian pernicious anemia.

Some information relative to a possible role of the intrinsic factor may be afforded by the studies on the two patients with macrocytic anemia following gastrectomy (Cases 3 and 12). The patient (Case 3) on whom hematologic data were obtained resembled those with true pernicious failure in the failure to respond to the administration of the hexaglutamyl conjugate. Neither of the gastrectomy patients excreted increased amounts of pteroylglutamic acid when they were given the conjugate. These results indicate that the stomach may be concerned with the liberation of the conjugate and lead to the suggestion that the intrinsic factor, although not itself capable of converting the hexaglutamyl conjugate to the free form,<sup>6</sup> may act enzymatically as an anti-inhibitor.

At this time it is possible only to speculate on the reasons why persons with pernicious anemia in liver extract-induced remission excrete larger amounts of pteroylglutamic acid than those in relapse, either when they are receiving a standard diet or during administration of relatively large amounts of a naturally occurring conjugate of the vitamin. The increased excretion rate observed in remission may be due either to a lessened state of tissue deficiency or to more efficient liberation of the vitamin from its conjugated form. However, both hypotheses may be true, since correction of a tissue deficiency by means of refined liver extract, containing very small quantities of pteroylglutamic acid, implies more effective utilization of natural forms of the vitamin. It should not be assumed, however, that the activity of liver extract in bringing about remission in pernicious anemia is limited to enhancing the availability of dietary sources of pteroylglutamic acid. The observation that pteroylglutamic acid, administered either orally or parenterally, is not equally effective in inducing remissions in all patients with pernicious anemia and that it may have no effect on the combined system degeneration frequently associated with this disease<sup>35</sup> indicates that the action of liver extract is more complex than simply to relieve a conditioned nutritional deficiency of pteroylglutamic acid. The rapidity of therapeutic response to the injection of refined liver extract exhibited by severely ill patients is quite unrelated to their dietary intake and must be attributed to a direct effect upon tissue metabolism. The observations of many investigators of chronic nutritional deficiency states demonstrate that replacement therapy even with excessive amounts of the

deficient nutritive may be ineffective or only partially effective in re-establishing normal cellular metabolism. Moreover, the protean manifestations of pernicious anemia, and in particular the variable degree of central nervous system involvement found in patients with this disease, suggest that a complex type of cellular enzyme disturbance may exist and that the action of the liver principle in restoring normal pteroylglutamic acid metabolism constitutes but one of its therapeutic effects.

#### SUMMARY AND CONCLUSIONS

Pteroylglutamic acid (folic acid) and its hexaglutamyl conjugate were given orally to sixteen patients. The series included nine patients with pernicious anemia in relapse, two with macrocytic anemia following gastrectomy, one with chronic liver disease associated with macrocytic anemia, one with macrocytic anemia believed to be due to nutritional deficiency, and three with pernicious anemia in liver extract induced remission.

The hematopoietic responses were observed and the urinary excretion of pteroylglutamic acid was measured during the administration of the conjugated and free vitamin.

Three patients with pernicious anemia in relapse and one patient with macrocytic anemia following gastrectomy exhibited little or no evidence of erythrocyte regeneration when given the hexaglutamyl conjugate in a yeast concentrate containing strong conjugase inhibiting activity. Significant responses occurred when these patients received either equivalent doses of synthetic pteroylglutamic acid or the yeast concentrate after incubation with an enzyme preparation which liberated the free vitamin from its conjugated form.

Two patients with pernicious anemia in relapse had definite hematopoietic responses when they were given the equivalent of 5 mg. daily of pteroylglutamic acid as the hexaglutamyl conjugate in a yeast concentrate containing relatively little enzyme inhibitor.

The patient with probable nutritional macrocytic anemia showed a sub-optimal response to the administration of the yeast concentrate with strong conjugase inhibiting activity.

Determinations made prior to the institution of therapy revealed that the urinary excretion of pteroylglutamic acid was lower in patients with macrocytic anemia than in normal subjects or patients with pernicious anemia in liver extract-induced remission.

In patients with pernicious anemia, postgastrectomy macrocytic anemia, and liver disease, the urinary excretion of free vitamin after the oral administration of conjugate with large amounts of inhibitor was much less than the level obtained in normal subjects. One patient with probable nutritional macrocytic anemia excreted the vitamin in amounts comparable to normal subjects.

In one patient with pernicious anemia previously untreated and in another who had been receiving treatment with pteroylglutamic acid without production of complete remission, the simultaneous administration for short periods of refined liver extract by intramuscular injection and the hexaglutamyl con-

jugate by mouth was not followed by increased pteroylglutamic acid excretion above the levels which had been observed when the conjugate was given alone. On the other hand, the administration of conjugated vitamin to three patients with pernicious anemia in liver extract-induced remission was followed by the excretion of the free material in amounts approximately equal to those obtained in normal subjects.

It is concluded that patients with pernicious anemia in relapse and post-gastrectomy macrocytic anemia cannot readily utilize for hematopoiesis a naturally occurring conjugate of pteroylglutamic acid although they respond promptly to administration of the free vitamin. This defect is not absolute and varies in different patients. Inability to utilize the conjugated vitamin appears to depend, at least in part, upon a conjugase inhibiting substance present in natural sources of conjugate.

Determination of the urinary excretion of pteroylglutamic acid after conjugate administration provides an index of the ability to liberate the free vitamin from its conjugated form.

One of the pharmacologic actions of the liver principle may be the correction of the metabolic defect in the utilization of naturally occurring conjugated forms of pteroylglutamic acid.

#### REFERENCES

1. Doan, C. A.: Folic Acid (Synthetic Lactobacillus Casei Factor), an Essential Pan-hematopoietic Stimulus; Experimental and Clinical Studies, *Am. J. M. Sc.* 212: 257, 1946.
2. Bird, O. D., Bressler, B., Brown, R. A., Campbell, C. J., and Emmett, A. D.: The Microbiological Assay of Vitamin B<sub>12</sub> Conjugate, *J. Biol. Chem.* 159: 631, 1945.
3. Pfaffner, J. J., Calkins, D. G., Bloom, E. S., O'Dell, B. L.: On the Peptide Nature of Vitamin B<sub>12</sub> Conjugate From Yeast, *J. Am. Chem. Soc.* 68: 1392, 1946.
4. Stokstad, E. L. R., and others: Conference on Folic Acid, New York Academy of Science, May 29, 1946; *Nutrition Rev.* 4: 199, 1946.
5. Bethell, F. H., Swendseid, M. E., Bird, O. D., Meyers, M. C., Andrews, G. A., and Brown, R. A.: Observations on the Utilization of Folic Acid (Vitamin B<sub>12</sub>) and Vitamin B<sub>12</sub> Conjugate by Persons With Pernicious Anemia and Macrocytic Anemia Following Gastrectomy, *Univ. Hosp. Bull., Ann Arbor* 12: 42, 1946.
6. Welch, A. D., Heinle, R. W., Nelson, E. M., and Nelson, H. V.: Ineffective Utilization of Conjugated Pteroylglutamic (Folic) Acid in Pernicious Anemia, *J. Biol. Chem.* 164: 787, 1946.
7. Castle, W. B.: The Etiology of Pernicious Anemia and Related Macrocytic Anemias, *Ann. Int. Med.* 7: 2, 1933.
8. Strauss, M. B., and Castle, W. B.: The Nature of the Extrinsic Factor of the Deficiency State in Pernicious Anemia and in Related Macrocytic Anemias. Activation of Yeast Derivatives With Normal Human Gastric Juice, *New England J. Med.* 207: 55, 1932.
9. Wills, L., and Evans, B. D. F.: Tropical Macrocytic Anaemia; Its Relation to Pernicious Anaemia, *Lancet* 2: 416, 1938.
10. Wills, L., and Stewart, A.: Experimental Anaemia in Monkeys, With Special Reference to Macrocytic Nutritional Anaemia, *Brit. J. Exper. Path.* 16: 444, 1935.
11. Day, P. L., Langston, W. C., and Shukers, C. F.: Leukopenia and Anemia in the Monkey Resulting From Vitamin Deficiency, *J. Nutrition* 9: 637, 1935.
12. Day, P. L., Langston, W. C., and Darby, W. J.: Failure of Nicotinic Acid to Prevent Nutritional Cytopenia in the Monkey, *Proc. Soc. Exper. Biol. & Med.* 38: 860, 1938.
13. Day, P. L., Mims, V., Totter, J. R., Stokstad, E. L. R., Hutchings, B. L., and Sloan, N. H.: The Successful Treatment of Vitamin M Deficiency in the Monkey With Highly Purified Lactobacillus Casei Factor, *J. Biol. Chem.* 157: 423, 1945.
14. Wilson, H. E., Saslaw, S., and Doan, C. A.: The Effect of Folic Acid (Lactobacillus Casei Factor) in Nutritional Hematopenia of Monkeys, *J. Lab. & Clin. Med.* 31: 631, 1946.
15. Hogan, A. G., and Parrott, E. M.: Anemia in Chicks Caused by a Vitamin Deficiency, *J. Biol. Chem.* 132: 507, 1940.

16. Pfiffner, J. J., Binkey, S. B., Bloom, E. S., Brown, R. A., Bird, O. D., Emmett, A. D., Hogan, A. G., and O'Dell, B. L.: Isolation of Antianemia Factor (Vitamin B<sub>12</sub>) in Crystalline Form From Liver, *Science* 97: 404, 1943.
17. Daft, F. S., and Sebrell, W. H.: Sulfonamides and Vitamin Deficiencies, *Vitamins & Hormones* 3: 58-63, 1945.
18. Watson, J., and Castle, W. B.: Nutritional Macrocytic Anemia, Especially in Pregnancy; Response to a Substance in Liver Other Than That Effective in Pernicious Anemia, *Am. J. M. Sc.* 211: 513, 1946.
19. Wintrobe, M. M.: Antianemic Effect of Yeast in Pernicious Anemia, *Am. J. M. Sc.* 197: 286, 1939.
20. Spies, T. D.: Folic Acid for Macrocytic Anemia in Relapse, *J. A. M. A.* 130: 474, 1946.
21. Darby, W. J., and Jones, E.: Treatment of Sprue With Synthetic Lactobacillus Casei Factor (Folic Acid, Vitamin M), *Proc. Soc. Exper. Biol. & Med.* 60: 259, 1945.
22. Moore, C. V., Bierbaum, O. S., Welch, A. D., and Wright, L. D.: The Activity of Synthetic Lactobacillus Casei Factor ("Folic Acid") as an Antipernicious Anemia Substance. I. Observations on Four Patients: Two With Addisonian Pernicious Anemia, One With Nontropical Sprue, and One With Pernicious Anemia of Pregnancy, *J. LAB. & CLIN. MED.* 30: 1056, 1945.
23. Goldsmith, G. A.: The Effect of Folic Acid on the Blood Picture in Human Macrocytic Anemia, *Federation Proc.* 5: 232, 1946.
24. Zuelzer, W. W., and Ogden, F. N.: Megaloblastic Anemia in Infancy, *Am. J. Dis. Child.* 71: 211, 1946.
25. Sharp, E. A., Vonder Heide, E. C., and Wolter, J. G.: Preliminary Clinical Observations on the Antianemia Vitamin B<sub>12</sub> (Yeast Concentrate), *J. A. M. A.* 124: 734, 1944.
26. Sharp, E. A., and Vonder Heide, E. C.: Failure to Induce Remission of Relapsed Pernicious Anemia by Oral Administration of Vitamin B<sub>12</sub> Concentrates, *Proc. Central Soc. Clin. Research* 17: 41, 1944.
27. Castle, W. B., Ross, J. B., Davidson, C. S., Burchenal, J. H., Fox, H. F., and Ham, T. H.: Extrinsic Factor in Pernicious Anemia; Ineffectiveness of Purified Casein and of Identified Components of the Vitamin B Complex, *Science* 100: 81, 1944.
28. Vilter, C. F., and Spies, T. D.: Quoted by Castle and associates.<sup>27</sup>
29. Watson, C. J., Schwartz, S., Sborov, V., and Bertie, E.: Studies of Urobilinogen; a Simple Method for Quantitative Recording of Ehrlich Reaction as Carried Out With Urine and Feces, *Am. J. Clin. Path.* 14: 605, 1944.
30. Angier, R. B., and others: Synthesis of a Compound Identical With the Lactobacillus Casei Factor Isolated From Liver, *Science* 102: 227, 1945.
31. Angier, R. B., and others: The Structure and Synthesis of the Liver Lactobacillus Casei Factor, *Science* 103: 667, 1946.
32. Swendseid, M. E., Bird, O. D., Brown, R. A., and Bethell, F. H.: Metabolic Function of Pteroylglutamic Acid and Its Hexaglutamyl Conjugate. II. Urinary Excretion Studies on Normal Subjects. Effect of a Conjugate Inhibitor, *J. LAB. & CLIN. MED.* 32: 23, 1947.
33. Bird, O. D., Robbins, M., Vandenbelt, J. M., and Pfiffner, J. J.: Observations on Vitamin B<sub>12</sub> Conjugase From Hog Kidney, *J. Biol. Chem.* 163: 649, 1946.
34. Bethell, F. H., and Goldhamer, S. M.: Standards for Maximum Reticulocyte Values Following Ventriculin and Intravenous Liver Extract Therapy in Pernicious Anemia, *Am. J. M. Sc.* 186: 480, 1933.
35. Vilter, C. F., Vilter, R. W., and Spies, T. D.: The Occurrence of Combined System Disease in Persons With Pernicious Anemia During Treatment With the Lactobacillus Casei Factor (Folic Acid), *Proc. Central Soc. Clin. Research* 19: 26, 1946.

# METABOLIC FUNCTION OF PTEROYLGLUTAMIC ACID AND ITS HEXAGLUTAMYL CONJUGATE

## II. URINARY EXCRETION STUDIES ON NORMAL PERSONS. EFFECT OF A CONJUGASE INHIBITOR

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THE evidence that pteroylglutamic acid by virtue of its role in erythrocytogenesis<sup>1</sup> is an essential factor in human nutritional economy gives emphasis to a study of the metabolic function of this vitamin. Since pteroylglutamic acid occurs in plant and animal tissues in a conjugated form,<sup>2, 3</sup> such a study must necessarily attempt to delineate the physiologic significance of conjugate in relation to free vitamin and the factors governing conjugate cleavage.

The present communication is concerned with the urinary excretion levels of pteroylglutamic acid (PGA) on normal subjects after the administration of the vitamin both in the free form and as yeast concentrates of the hexaglutamyl conjugate. It was found that there are substances present in certain yeast extracts which lower the expected vitamin excretion rate following conjugate administration, results that are interpreted as evidence of the *in vivo* action of inhibitors on conjugate conversion.

Bird and associates<sup>4</sup> have reported from the results of their *in vitro* studies on the cleavage of hexaglutamyl conjugate by the enzyme pteroylglutamic acid (PGA) conjugase that there was a substance present in natural sources (that is, yeast extracts) which caused enzyme inhibition.

The conjugate of pteroylglutamic acid used in this investigation was the peptide, pteroylhexaglutamylglutamic acid. This conjugate has been isolated in crystalline form from yeast,<sup>5</sup> and either it or a conjugate of similar microbiologic activity is widely distributed in both plant and animal tissues.<sup>3</sup> For example, we have found<sup>6</sup> that over 90 per cent of pteroylglutamic acid in the leucocytes and their precursors occur as this type of conjugate. The only other known conjugate is pteroyltriglutamic acid or fermentation factor.<sup>7, 8</sup> Spies<sup>9</sup> has reported a single patient with pernicious anemia in relapse who responded to the intramuscular injection of this material. However, there is no evidence on the basis of differential microbiologic assays<sup>6</sup> that this conjugate has a general natural distribution, and hence it would appear that it may not be of practical nutritional significance.

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\*The hexaglutamyl conjugate when tested microbiologically has a *Lacto-bacillus casei* activity equivalent to *Streptococcus faecalis* activity; after conjugase treatment *L. casei* and *Str. faecalis* activities are also equivalent but are increased. Pteroyltriglutamic acid has an *L. casei* activity higher than its *Str. faecalis* activity; after conjugase treatment *Str. faecalis* activity increases while *L. casei* activity remains the same.



## EXPERIMENTAL

Seven normal individuals took part in the study. Most of them received separately two hexaglutamyl conjugate concentrates as well as the free vitamin in daily doses equivalent to 4 mg. of pteroylglutamic acid. Each preparation was administered orally for two consecutive days and the treatment periods were spaced at intervals of one to four weeks.

The two hexaglutamyl conjugate concentrates used in this study were prepared from yeast. One of the concentrates<sup>\*</sup> was a much purer preparation than the other and contained no conjugase inhibitor. The other concentrate was a norite eluate of yeast and contained conjugase inhibitor. The inhibitor activity of this concentrate relative to the amount of pteroylglutamic acid present as determined by in vitro tests<sup>1</sup> was approximately one-third the concentration found in the original yeast. To split the conjugate present in this concentrate required thirty-six times as many PGA conjugase enzyme units as were necessary to free an equivalent amount of vitamin from crystalline hexaglutamyl conjugate.

The pteroylglutamic acid content of the urine was determined by microbiologic assay using *L. casei*. In some instances analyses were made following treatment of the urine with PGA conjugase from hog kidney to determine whether the vitamin was excreted in the conjugate form. The methods used for the assay and treatment with conjugase have been described.<sup>3</sup>

## RESULTS

Tables have been set up showing the urinary excretion levels of PGA before and during the daily administration of 4 mg. of pteroylglutamic acid or concentrates of hexaglutamyl conjugate containing the equivalent of 4 mg. of pteroylglutamic acid.

No evidence for the excretion of the vitamin as the hexaglutamyl conjugate was obtained in any of these experiments, including those where the conjugate was administered as such. These results are not included in the tables.

The values for PGA excretion on unsupplemented diets (averages of 2.5 and 2.9 micrograms on consecutive days) are similar to those studies already reported.<sup>10-12</sup> When pteroylglutamic acid was given, about one-third of the 4 mg. dose was excreted in the urine.

When a concentrate of hexaglutamyl conjugate free from conjugase inhibitor was administered, the urinary PGA excretion levels (averages of 1,193 and 1,412  $\mu$ g on consecutive days, Table II) were comparable to those obtained when an equivalent amount of the vitamin was given in the free form (averages of 1,266 and 1,288  $\mu$ g on consecutive days, Table I). However, the administration of conjugate concentrates containing conjugase inhibitor resulted in urinary PGA excretion values (averages of 153 and 244  $\mu$ g on consecutive days, Table II) which were definitely lower than when the free vitamin or conjugate without inhibitor was given.

\*Obtained from Dr. J. J. Pflüger of the Parke, Davis & Company Laboratories.

TABLE I. URINARY PGA EXCRETION IN NORMAL SUBJECTS BEFORE AND DURING ADMINISTRATION OF 4 MG. PTEROYLGLUTAMIC ACID

SUBJECT	BEFORE TREATMENT ( $\mu$ G PGA PER 24 HR.)		DURING TREATMENT ( $\mu$ G PGA PER 24 HR.)	
	DAY I	DAY II	DAY I	DAY II
He	3.1	2.1	1,381	1,364
Ro	2.3	2.1	1,235	1,221
Ov	2.3	2.9	1,112	1,144
Br	--	3.9	1,184	1,371
Fe	2.9	3.4	1,386	1,374
Bi	2.4	4.1	1,298	1,253
Average	2.5	2.9	1,266	1,288

TABLE II. URINARY PGA EXCRETION IN NORMAL SUBJECTS DURING ADMINISTRATION OF HEXAGLUTAMYL CONJUGATE CONCENTRATES CONTAINING EQUIVALENT OF 4 MG. PGA

SUBJECT	DURING TREATMENT WITH HEXAGLUTAMYL CONJUGATE ( $\mu$ G PGA PER 24 HR.)		DURING TREATMENT WITH HEXAGLUTAMYL CONJUGATE CONTAINING INHIBITOR ( $\mu$ G PGA PER 24 HR.)	
	DAY I	DAY II	DAY I	DAY II
Ro	--	--	155	310
Ov	--	--	260	288
He	2,087	2,184	151	351
Br	1,654	1,807	201	238
Fe	639	1,025	52	143
Bi	517	797	53	163
Sw	1,068	1,248	200	218
Average	1,193	1,412	153	244

TABLE III. URINARY PGA EXCRETION IN NORMAL SUBJECTS DURING ADMINISTRATION OF THE VITAMIN OR ITS HEXAGLUTAMYL CONJUGATE WITH A CONJUGASE INHIBITOR SOURCE

SUBJECT	DAILY TREATMENT	URINARY EXCRETION OF PGA ( $\mu$ G PGA PER 24 HR.)	
		DAY I	DAY II
He	Conjugate (equivalent to 4 mg. PGA) + 30 Gm. Difco yeast extract	110	--
Br	Conjugate (equivalent to 4 mg. PGA) + 30 Gm. Difco yeast extract	76	156
Bi	4 mg. PGA + 30 Gm. Difco yeast extract	1,190	1,030
Sw	4 mg. PGA + 30 Gm. Difco yeast extract	1,350	1,460

To study further the effect of conjugase inhibitor, 30 Gm. of Difco yeast extract as a source of inhibitor was added to the purified conjugate concentrate (the equivalent of 4 mg. of PGA). In two subjects, PGA excretion levels (110  $\mu$ g and an average of 116  $\mu$ g per twenty-four hours, Table III) were greatly reduced below the values obtained when the conjugate was given alone.

The urinary PGA excretion levels were also determined during the administration of the free vitamin form together with a source of conjugase inhibitor. The values for each of two subjects (average 1,110 and 1,465  $\mu$ g per twenty-four hours, Table III) were similar to those obtained when the free vitamin alone was administered (Table I).

## DISCUSSION

It has been demonstrated that a hexaglutamyl conjugate concentrate containing PGA conjugase inhibitor when administered to normal subjects results in a lower excretion of PGA than when an equivalent amount of a purer conjugate without inhibitors is given. Moreover, when a natural product containing inhibitor is given with this purer conjugate, urinary excretion levels of PGA are greatly reduced. These data are interpreted as evidence that the PGA conjugase inhibitor can act *in vivo* on conjugase enzyme systems to reduce the cleavage rate of the hexaglutamyl conjugate and hence to decrease subsequent urinary excretion of the free vitamin. PGA conjugase inhibitor does not affect the excretion level of pteroylglutamic acid when the vitamin is administered in the free form.

It is very possible that the inhibition of the conjugase enzyme system by a dietary source material as observed in this study occurs in the gastrointestinal tract and that at least one of the resultant effects is on the rate of absorption of the vitamin. Either this inhibitor or an endogenous product of similar nature may also be active in internal metabolic cycles to hold the greater portion of the body pteroylglutamic acid content as a conjugate.

While factors affecting *in vitro* enzyme experiments do not necessarily influence the complex enzyme systems in the body, the possibility must be considered that the same substance inhibits PGA conjugases *in vivo* and hog kidney PGA conjugase *in vitro*.<sup>4</sup>

The implications of this *in vivo* action of PGA conjugase inhibitor are far-reaching. The hexaglutamyl conjugate, the type of compound which serves for the most part as the nutritional source of pteroylglutamic acid, occurs naturally in association with conjugase inhibitor (results of *in vitro* tests). Such substances as liver, yeast, and spinach, which contain relatively large amounts of conjugate, are also high in inhibitor content.<sup>13</sup> Therefore, from a nutritional and metabolic standpoint, studies involving the role of hexaglutamyl conjugate cannot be considered apart from conjugase inhibitor.

Wright and Welch<sup>10</sup> have stated that, in contrast to other members of the vitamin B group, only a small percentage of the folic acid estimated to be contained in the diet is excreted by normal individuals. It is probable that the data reported here may offer an explanation for this seeming anomaly as regards folic acid in that the action of conjugase inhibitor reduces the rate of cleavage of conjugate to the free vitamin, the form in which pteroylglutamic acid is excreted.

It has been reported that in pernicious anemia there is a lessened ability to utilize conjugated as compared with free pteroylglutamic acid for hemato-poiesis. These experiments with normal subjects led to the consideration that in patients with pernicious anemia the PGA conjugase inhibitor in the hexaglutamyl conjugate concentrates<sup>14</sup> might be a factor influencing conjugate utilization, and evidence supporting this idea has been presented in the preceding paper.<sup>15</sup>

## SUMMARY

The excretion values of pteroylglutamic acid have been determined for normal persons before and during the daily administration of 4 mg. doses of the vitamin in the free and conjugated forms.

It has been found that for the normal subject given hexaglutamyl conjugate, the urinary excretion levels of pteroylglutamic acid are dependent upon the presence of an exogenous source of conjugase inhibitor. Administering hexaglutamyl conjugate without inhibitor results in PGA excretion levels similar to those obtained following treatment with the free vitamin. Simultaneous administration of conjugase inhibitor and hexaglutamyl conjugate results in lower PGA excretion levels.

## REFERENCES

1. Berry, L. J., and Spies, T. D.: The Present Status of Folic Acid, *Blood* 1: 271, 1946.
2. Binkley, S. B., Bird, O. D., Bloom, E. S., Brown, R. A., Calkins, D. G., Campbell, C. J., Emmett, A. D., and Pfiffner, J. J.: On the Vitamin B<sub>12</sub> Conjugate in Yeast, *Science* 100: 36, 1944.
3. Bird, O. D., Bressler, B., Brown, R. A., Campbell, C. J., and Emmett, A. D.: The Microbiological Assay of Vitamin B<sub>12</sub> Conjugate, *J. Biol. Chem.* 159: 631, 1945.
4. Bird, O. D., Robbins, M., Vandenbelt, J. M., and Pfiffner, J. J.: Observations on Vitamin B<sub>12</sub> Conjugase From Hog Kidney, *J. Biol. Chem.* 163: 649, 1946.
5. Pfiffner, J. J., Calkins, D. G., O'Dell, B. L., Bloom, E. S., Brown, R. A., Campbell, C. J., and Bird, O. D.: Isolation of an Antianemia Factor (Vitamin B<sub>12</sub> Conjugate) in Crystalline Form From Yeast, *Science* 102: 228, 1945.
6. Unpublished data.
7. Hutchings, B. L., Stokstad, E. L. R., Bohonos, N., and Slobodkin, N. H.: Isolation of a New *Lactobacillus Casei* Factor, *Science* 99: 371, 1944.
8. Lampen, J. O., and Jones, M. J.: The Antagonism of Sulfonamides by Pteroylglutamic Acid and Related Compounds, *J. Biol. Chem.* 164: 485, 1946.
9. Spies, T. D.: The Activity of Another *Lactobacillus Casei* Factor in Macrocytic Anemia, *South. M. J.* 39: 634, 1946.
10. Wright, L. D., and Welch, A. D.: The Production of Folic Acid by Rat Liver in Vitro, *Science* 98: 179, 1943.
11. Johnson, B. C., Hamilton, T. S., and Mitchell, H. H.: The Excretion of "Folic Acid" Through the Skin and in the Urine of Normal Individuals, *J. Biol. Chem.* 159: 425, 1945.
12. Denco, C. W., Grundy, W. E., Porter, J. W., Berryman, G. H., Freidemann, T. E., and Youmans, J. B.: The Excretion of B-Complex Vitamins in the Urine and Feces of Seven Normal Adults, *Arch. Biochem.* 10: 33, 1946.
13. Unpublished data.
14. Bethell, F. H., Swendseid, M. E., Bird, O. D., Meyers, M. C., Andrews, G. A., and Brown, R. A.: Observations on the Utilization of Folic Acid (Vitamin B<sub>12</sub>) and Vitamin B<sub>12</sub> Conjugate by Persons With Pernicious Anemia and Macrocytic Anemia Following Gastrectomy, *Univ. Hosp. Bull., Ann Arbor* 12: 42, 1946.
15. Bethell, F. H., Meyers, M. C., Andrews, G. A., Swendseid, M. E., Bird, O. D., and Brown, R. A.: Metabolic Function of Pteroylglutamic Acid and Its Hexaglutamyl Conjugate. I. Hematologic and Urinary Excretion Studies on Patients With Macrocytic Anemia, *J. LAB. & CLIN. MED.* 32: 3, 1947.

## THE EFFECT OF THE METHYLATED XANTHINES ON THE CLOTTING TIME OF THE BLOOD

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THE methylated xanthines, in one form or another, have been in use for over half a century. In the last two decades they have been used very extensively. In spite of occasional adverse criticism, there is good reason to think, from accumulated experimental and clinical evidence, that they are of very definite therapeutic value. Any contraindication for their use should be examined carefully and critically.

It has been reported recently<sup>1</sup> that the methylated xanthines in experimental animals increased the prothrombin level of the blood and increased its coagulability. It would be assumed that if any such action resulted from their clinical use, abundant evidence of such an effect would have been previously observed and reported. On the contrary, it has been our impression, at least, that the patients using one or another of the xanthines over a long period of time had a longer life expectancy, not a shorter. Our experience has been that of Fahr,<sup>2</sup> who has had a long and carefully observed experience with these preparations, that heart failure is apparently postponed.

We have accumulated a great deal of data which we will not attempt to present here in detail, but we will choose only enough to show what we consider very clear evidence that the methylated xanthines have no effect in prolonging the clotting time of the blood.

As a measure of the coagulability of the blood, we determined the clotting time, the response of the subject to intravenous heparin, and the prothrombin level. Clotting times were determined by the capillary tube method. The heparin tolerance test has been adequately described and its significance discussed by de Takats.<sup>3</sup> By his technique, an initial clotting time is determined and then 10 mg. of heparin are injected intravenously. Clotting times are determined at ten-minute intervals until the initial level of the blood is again established.

Prothrombin times were determined on the whole plasma and the diluted (12.5 per cent) plasma, using the technique of Quick. In order to establish the normal prothrombin time for our laboratory, control determinations were made on ten normal individuals. By our technique, the normal time for whole plasma was found to vary between 11 and 12.5 seconds, with a mean of 12; while the time for dilute plasma, with two exceptions, was between 32 and 38 seconds, with a mean of 34. In dogs the normal prothrombin time for whole plasma was 6 seconds and for the 12.5 per cent plasma it was 13 to 17 seconds. The results are the average of duplicate or triplicate determinations.

For the study of the immediate effect of the methylated xanthines upon clotting activity, normal individuals were admitted to the hospital, and the

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coagulability of the blood was determined before and after the administration of the drug. The patients were placed upon a routine, general ward diet. They were treated as ambulatory patients and permitted the freedom of the ward. During the first week in the hospital, daily heparin curves were taken and one or more prothrombin determinations were made. The patients then were given one of the xanthines, usually aminophyllin, either orally or parenterally, and the heparin curves and prothrombin determinations were made at various intervals.

To observe the effect of intramuscular injections, two normal patients were hospitalized and kept under control conditions for the course of the observations. Because of variations of clotting time and the course of the heparin curve, control readings were made for six days. Only two of the curves are plotted in each case. The curves for the other days lay between these two.

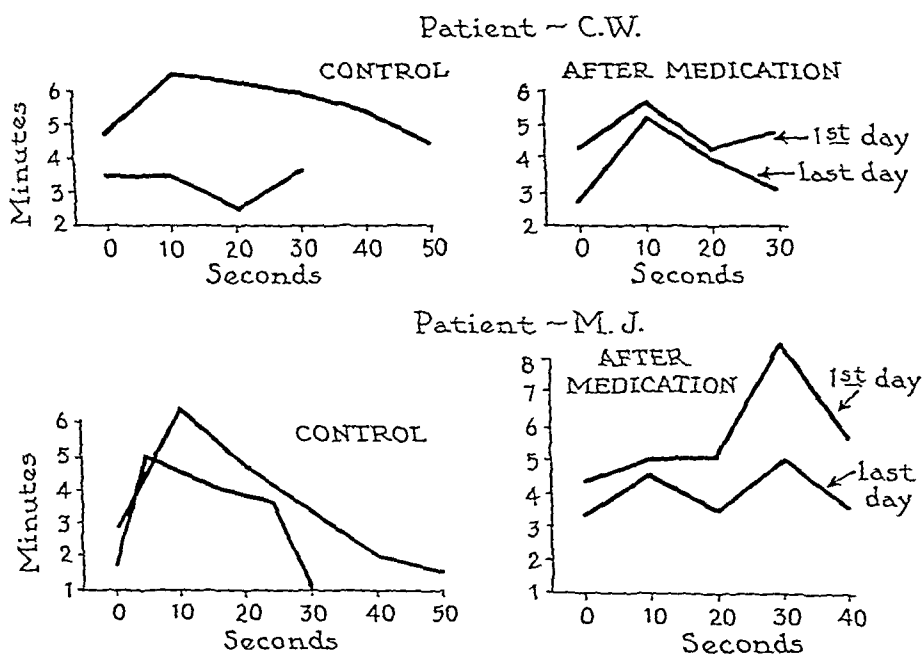


Fig. 1.—Heparin curves before and after the administration of intramuscular aminophyllin, grains  $7\frac{1}{2}$ , on two patients.

Similarly, after administration of the drug, only the curves of the first and the last day are plotted. These are shown in Fig. 1. In the first patient (C.W.) the control prothrombin readings were 12 seconds for the whole plasma and 33 seconds for the dilute plasma. Prothrombin readings were taken every hour for three hours after the first injection of aminophyllin. These readings were, respectively, 12 seconds for the whole plasma and 34 seconds for the dilute plasma; 12 and 34; 12 and 35; and 12 and 34. At the end of five days the prothrombin times were 12 seconds and 34 seconds.

The second patient (M.J.) showed a control prothrombin time of 12 seconds for the whole plasma and 34 seconds for the dilute plasma. The same readings were obtained at the end of six days.

Two normal patients were given intravenous aminophyllin every eight hours for three doses. The coagulation time and the heparin curve are shown in Fig. 2. The prothrombin times did not change from the controls at the end of the three-day period.

Aminophyllin,  $7\frac{1}{2}$  grains intravenously, was also administered to three individuals who were not normal, and the prothrombin levels were determined at hourly intervals for three hours and again at six hours after administration. Two of the patients had been on theobromine calcium salicylate for four weeks; the third was a patient with lymphatic leucemia and had had no medication. The results are shown in Table I.

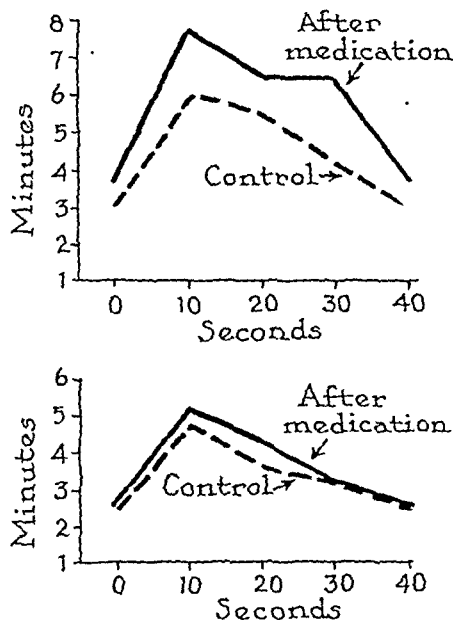


Fig. 2.—Heparin curves before and after the administration of intravenous aminophyllin, grains  $7\frac{1}{2}$ , on two patients.

Aminophyllin was given to three normal patients in 3 grain doses, three times daily for from fifteen to twenty-eight days. Control prothrombin levels were taken as were heparin curves; prothrombin levels and heparin curves were repeated at intervals of from two days to one week during the administration of the drug. The results were consonant with those obtained in the previous patients and will not be shown in detail. Similar determinations were made on nine patients with cardiac disease who were ambulatory and uncontrolled. These patients took theobromine calcium salicylate,  $7\frac{1}{2}$  grains daily by mouth. Normal coagulation times, normal heparin curves, and normal prothrombin levels were present throughout the experiment.

Further experiments were done upon seven dogs. One dog weighing 13 kilograms was given 15 grains of aminophyllin in one dose and was observed for three days. It suffered no ill effects from the large dose. The prothrombin times (in seconds) with the whole and dilute plasma are given in Table II. The coagulation time and heparin curves showed no significant changes from the controls of the previous two days.

TABLE I

	J., AGE 64 (CORONARY OCCLUSION)		B., AGE 42 (CORONARY OCCLUSION)		S., AGE 54 (LYMPHATIC LEUCEMIA)	
	WHOLE PLASMA (SEC.)	DILUTE PLASMA (SEC.)	WHOLE PLASMA (SEC.)	DILUTE PLASMA (SEC.)	WHOLE PLASMA (SEC.)	DILUTE PLASMA (SEC.)
Control	11.5	35	11	34	11	60
1 hour	11	35	12	35	11.5	65
2 hours	12	36	12	35	11	67
3 hours	11.5	38	12	38	11	64
6 hours	11	37	11	38	11	65

TABLE II

	WHOLE PLASMA (SEC.)	DILUTE PLASMA (SEC.)
1 hour	6	13
2 hours	6	14
3 hours	6	13
24 hours	6	14
48 hours	6	15
72 hours	6	14

Another dog weighing 11.3 kilograms was given 135 mg. of aminophyllin three times a day for two weeks. This dose was chosen in order to duplicate an experiment in which changes in prothrombin time were noted in such an experiment elsewhere. The first control reading and the heparin curve and the last heparin curve at the termination of the experiment are given in Fig. 3. The two prothrombin readings before the experiment were 6 and 15 seconds. During the experiment the prothrombin times varied from 6 and 15 seconds to 6 and 17 and were 6 and 16 on the last day. The other experiments upon dogs were repetitions of the clinical experiments and developed nothing different from the results shown. Aminophyllin was used in six experiments and theobromine calcium salicylate in one. The results throughout these experiments upon animals were in complete accord with those of Quick.<sup>5</sup>

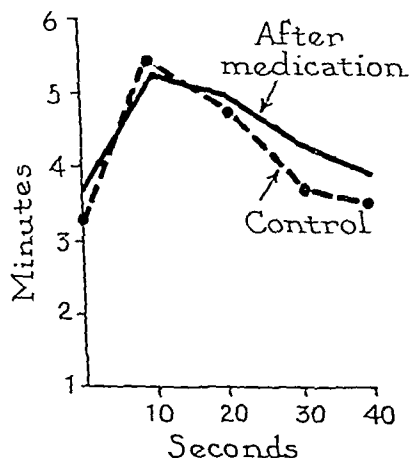


Fig. 3.

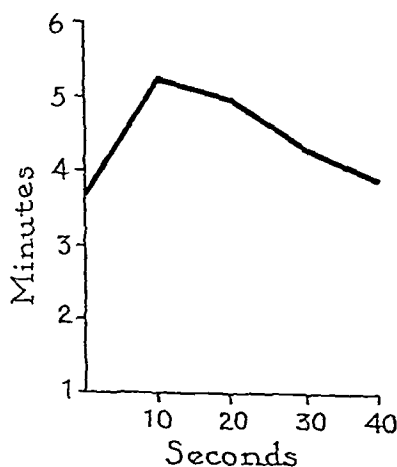


Fig. 4.

Fig. 3.—Dog given 135 mg. of aminophyllin three times a day for two weeks. Control heparin curve and heparin curve at termination of experiment.

Fig. 4.—Composite heparin curve derived from averages on patients who had taken methylxanthines for from ten days to twenty years.



In order to determine whether the prolonged administration of the methylated xanthines has any effect upon clotting activity, studies were made of twenty-five patients chosen at random who had been taking one or another of these preparations for from ten days to twenty years. The results are shown in Table III. Heparin curves were taken in each case. A composite curve is shown; it is based upon the arithmetical averages of each period after the administration of heparin (Fig. 4). The two extremes are shown in Fig. 5. In each case the patient had been taking aminophyllin for two years.

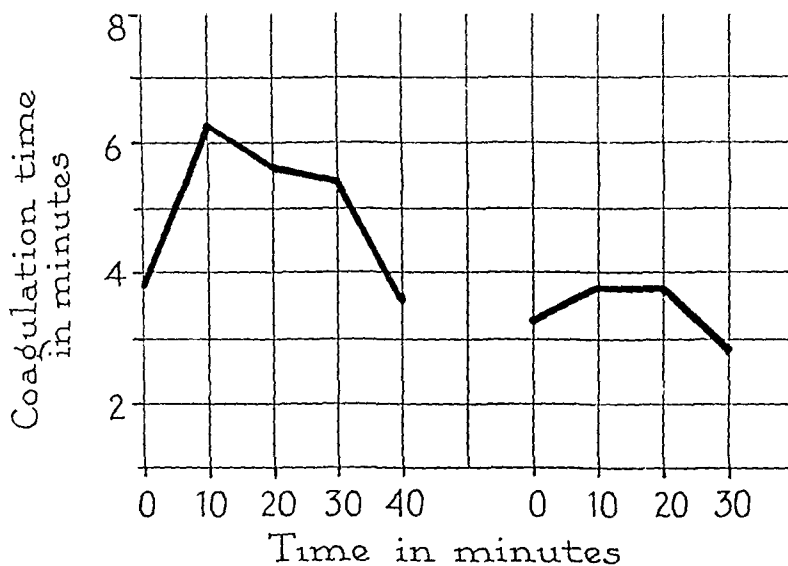


Fig. 5.—These two curves represent the two extremes on patients taking aminophyllin over long periods of time. In each case aminophyllin had been taken continuously for two years.

After the foregoing work was done, Scherf and Schlachman<sup>6</sup> reported a shortening of prothrombin time following the intravenous administration of aminophyllin and theobromine sodium acetate. Rieben,<sup>7</sup> on the other hand, reported that he was unable to confirm the work of Link showing the shortening of the prothrombin time to result from the administration of the methylated xanthines.

We are conscious of the conflicting and confusing reports which have appeared on this subject. A very ready recourse is available for determination of the effects of these drugs by following the advice of Hunter to Jenner—"Don't think. Try!" The determination of the coagulation time by the capillary tube method is easily done, requires no special apparatus, and is accurate for clinical purposes. Allowance must be made for normal, minor deviations from day to day, depending upon several variables. Determinations of prothrombin time admit of many errors. This is especially true when different modifications are used which introduce doubtful variables. It would add to the value of comparative studies if a standard method, such as that of Quick, were used. After all, however, the real point, as far as the methylated xanthines are concerned, is whether the coagulation of the blood is or is not hastened. This can readily be determined in any doctor's office.

TABLE III. CLOTTING ACTIVITY OF PATIENTS RECEIVING METHYLATED XANTHINES FOR LONG PERIODS OF TIME

PATIENT	PERIOD OF MEDICATION	CLOTTING TIME (MIN. AND SEC.)	PROTHROMBIN TIME	
			WHOLE PLASMA (SEC.)	DILUTE PLASMA (SEC.)
1	10 days	3	12	34
2	21 days	4	12	36
3	5 weeks	4	12	36
4	4 months	3, 20		
5	5 months	4, 15	12	36
6	6 months	3, 40	12.5	36
7	18 months	3, 30		
8	2 years	3, 15	12.5	34
9	2 years	3, 45	12	36
10	2.5 years	3, 50	11	33
11	3.5 years	4, 40		
12	3 years	5, 45	12.5	36
13	3 years	3, 00	12	35
14	3.5 years	4, 00	11	33
15	4 years	5	11.5	32
16	5 years	3, 45	12.5	34
17	5.6 years	3, 30		
18	6 years	3	11.5	33
19	6 years	3, 30		
20	7 years	3, 40		
21	8 years	6	12	34
22	10 years	2, 30		
23	14 years	4, 25		
24	16 years	3, 00	12.5	35
25	20 years	4	11.5	33

## SUMMARY

A series of experiments were carried out on patients and on animals to determine whether the prothrombin time, the clotting time of the blood, and the heparin curve were in any way changed by the administration of the xanthine drugs. Prothrombin times were determined in the whole plasma and in the dilute plasma. No change was found by either method. Coagulation times were not affected. The heparin curves were either not affected or were improved.

We wish to express our indebtedness to Dr. Armand J. Quick for his kindly help and advice in the methods of determining prothrombin time and in perfecting our technique.

## REFERENCES

1. Field, J. B., Larsen, E. G., Spero, L., and Link, P.: Studies on the Hemorrhagic Sweet Clover Disease. XIV. Hyperprothrombinemia Induced by Methylxanthines and Its Effect on the Action of 3,3'-Methylenebis-(4-Hydroxycoumarin), *J. Biol. Chem.* 156: 725, 1944.
2. Fahr, G.: Personal communication.
3. de Takats, G.: Heparin Tolerance; a Test of the Clotting Mechanism, *Surg., Gynec. & Obst.* 77: 31, 1943.
4. Quick, A. J.: The Hemorrhagic Diseases and the Physiology of Hemostasis, Springfield, Ill., 1942, Charles C Thomas, Publisher, p. 312.
5. Quick, A. J.: Experimentally Induced Changes in the Prothrombin Level of the Blood. Quantitative Studies in Dogs Given Dicumarol. II. The Effect of Methylxanthines on Prothrombin per se and When Administered With Dicumarol, *J. Biochem.* 161: 33, 1945.
6. Scherf, D., and Schlachman, M.: The Effect of Methylxanthines on the Prothrombin Time and Coagulation of the Blood. *Am. J. M. Sc.* 212: 83, 1946.
7. Rieben, W. K.: Beobachtungen mit einer neuen Prothrombin Bestimmungsmethode, *Helvet. med. acta* 13: 295, 1946.

# PENICILLIN IN BEESWAX AND PEANUT OIL. A NEW PREPARATION WHICH IS FLUID AT ROOM TEMPERATURE: ABSORPTION AND THERAPEUTIC USE

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PENICILLIN is administered by intermittent intramuscular injections more frequently than by any other method. While continuous intravenous and intramuscular infusions may be best for maintaining high concentrations of the antibiotic over a long period of time, they are necessarily restricted to patients who are closely confined. Likewise, oral administration can be used only in the treatment of certain groups of patients, particularly in those with diseases that respond easily and quickly to penicillin. The major disadvantage of administering penicillin intramuscularly in aqueous solution is the necessity for repeating the injections at two- or three-hour intervals. Consequently, several attempts have been made to slow the absorption of penicillin after it is deposited within the muscles; the most successful of these attempts has been the incorporation of the antibiotic in beeswax and peanut oil by Romansky and Rittman.<sup>1</sup> A single injection of 1 c.c. of their preparation, containing 300,000 units of penicillin, is usually followed by therapeutically effective concentrations in the blood for four to twenty-four hours.<sup>2, 3</sup> There are certain technical difficulties, however, in the administration of this mixture when it is made according to the original formula. The material is in a solid state at room temperature, and in order to liquefy must be heated before it is injected.

We have studied a preparation containing pure penicillin G which is a modification of the original formula. Merek & Company, Inc., from whom it was obtained, state that the procedure employed in its manufacture "alters the physical state of the menstruum and substantially reduces the viscosity at all temperatures."<sup>4</sup> We found that this material became fluid within fifteen to thirty minutes after it was removed from the refrigerator to room temperature and that it was not necessary to heat it before it was administered. In the present paper we are reporting the results of our study of the absorption of this mixture and of its use in patients with various infections.

## STUDIES ON ABSORPTION

Both crystalline sodium penicillin G and purified calcium penicillin G were administered to an equal number of patients. We found no difference between the two salts, except that the sodium penicillin was lighter in color and became fluid more quickly at room temperature. Each cubic centimeter contained

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\*Stable at room temperature for at least six months.

300,000 units in refined peanut oil containing 4.8 per cent (W/V) of U. S. P. bleached beeswax. It was administered by using separate 18 or 20 gauge needles for withdrawal and for injection. It was found necessary that both the syringes and the needles be *absolutely* dry.

Twenty subjects without any evidence of impaired renal function or of cardiac or hepatic failure were given 300,000 units of the penicillin, contained in 1 c.c. of wax and oil, deep in the gluteal muscles. Eleven patients received the sodium salt and nine the calcium penicillin, but since no differences were observed in the serum concentrations obtained the two preparations will be considered together. Blood for penicillin determinations was taken at three-hour intervals for twelve hours starting twelve hours after injection in sixteen subjects and during the whole twenty-four hour period following injection in four patients. Serum concentrations of penicillin were determined by a modification of the method of Rammelkamp.<sup>5</sup> The results are shown in Table I. None of the patients had an assayable concentration of penicillin in the serum at twenty-four hours. Five subjects had significant concentrations at twenty hours, ten at eighteen hours, and fourteen at fifteen hours. All but two of the subjects had detectable concentrations of the antibiotic in their serum at twelve hours. Concentrations ranging from 0.625 to 5 units per cubic centimeter of serum were found in four subjects three to nine hours after injection. In two subjects concentrations of 1.25 and 0.156 units per cubic centimeter were found in the serum fifteen and thirty minutes, respectively, after injection.

TABLE I. SERUM CONCENTRATIONS OF PENICILLIN (UNITS PER CUBIC CENTIMETER) FOLLOWING SINGLE INTRAMUSCULAR INJECTIONS OF 300,000 UNITS (1 c.c.) OF PENICILLIN-BEESWAX-PEANUT OIL MIXTURE

PATIENT	3 HR.	6 HR.	9 HR.	12 HR.	15 HR.	18 HR.	21 HR.	24 HR.
9				0	0	0	0	0
15				0	0		0	0
11				1.25	0	0	0	0
12				0.312	0	0	0	0
16				0.039	0		0	0
8				0.156	0.078	0	0	0
10				0.312	0.039	0	0	0
17				0.625	0.078		0	0
18				0.312	0.078		0	0
2	2.5	1.25	1.25	1.25		0	0	0
1	1.25	0.625	0.625	0.625		0.039		0
7				0.625	0.156	0.039	0	0
3	5.0	1.25	1.25	1.25		0.078		0
4	5.0	5.0	5.0	5.0	2.5	0.625	0	0
13				1.25	0.156	0.078	0	0
6					0.625	0.078	0.078	0
5				2.5	0.625	0.625	0.312	0
14				5.0	0.078	0.078	0.078	0
19				0.625	0.312		0.039	0
20				0.625	0.156		0.039	0

Blood was taken on two occasions from one patient who was receiving daily injections of 300,000 units twenty-four hours after a preceding injection, and no penicillin was detectable.

Since significant concentrations of penicillin were found in the serum of most of the subjects studied for only twelve hours following the injection of 300,000 units, it was decided to study the absorption of 600,000 units of penicillin in wax and oil, contained in 2 c.c. of the mixture. Thirty-six subjects

were given either the sodium or the calcium preparation deep in the thigh or gluteal muscles. Blood for penicillin determination was taken at four-hour intervals thereafter for twenty-four hours in four subjects and at the same intervals starting twelve hours after injection in thirty-one patients. The results are shown in Table II. Twelve of the thirty-six subjects studied had assayable concentrations of penicillin in their serum at twenty-four hours, twenty at twenty hours, and twenty-nine at sixteen hours. In only two patients was there no evidence of penicillin in the serum at twelve hours. Concentrations of penicillin ranging from 0.312 to 10 units per cubic centimeter were found in the serum of five subjects four and eight hours after the injections. Two subjects had serum concentrations of 1.25 units per cubic centimeter thirty minutes after administration of the mixture.

Kirby and associates<sup>6</sup> found that a higher percentage of patients had detectable concentrations of penicillin in their blood at twenty-four hours following subcutaneous injection of 600,000 units of penicillin in beeswax and peanut oil than following intramuscular injection of a similar amount. Consequently, we gave twenty subjects 600,000 units of penicillin, contained in 2 c.c.

TABLE II. SERUM CONCENTRATIONS OF PENICILLIN (UNITS PER CUBIC CENTIMETER) FOLLOWING SINGLE INTRAMUSCULAR INJECTIONS OF 600,000 UNITS (2 C.C.) OF PENICILLIN-BEESWAX-PEANUT OIL MIXTURE

PATIENT	4 HR.	8 HR.	12 HR.	16 HR.	20 HR.	24 HR.
3			0	0	0	0
14	2.5	0.625	0	0	0	0
12	10.0	0.312	0.078	0	0	0
9			0.156	0	0	0
8			0.156	0	0	0
31			0.156	0	0	0
32			0.156	0	0	0
15	2.5	1.25	0.078	0.039	0	0
16			2.5	0.625	0	0
18			5.0	0.625	0	0
23			0.312	2.5	0	0
28			0.625	0.156	0	0
29			10.0	0.625	0	0
33			1.25	0.156	0	0
34			0.625	0.156	0	0
35			0.625	0.078	0	0
7			0.156	0.156	0.078	0
6			0.625	0.312	0.078	0
5			0.625	0.312	0.078	0
2			1.25	0.625	0.156	0
20			5.0	1.25	0.156	0
11			2.5	0.625	0.312	0
17			5.0	5.0	0.625	0
36			0.625	0.156	0.156	0
13	10.0	1.25	0.625	0.312	0.078	0.078
10			0.625	0.625	0.312	0.156
21			1.25	0.625	0.156	0.156
1			2.5	1.25	0.312	0.156
4			> 40.0	5.0	1.25	0.156
19			1.25	1.25	0.312	0.312
22			2.5	1.25	0.625	0.156
24			2.5	1.25	1.25	1.25
25			1.25	1.25	1.25	1.25
26			10.0	10.0	0.625	0.312
27			20.0	10.0	0.625	0.625
30			10.0	2.5	0.312	0.312

of wax and oil, subcutaneously. Blood was obtained at four-hour intervals for twelve hours starting twelve hours after injection in thirteen subjects and at four, eight, sixteen, twenty-four, and twenty-eight hours after injection in seven subjects. The results are shown in Table III. All but one of the patients had detectable concentrations of penicillin in the serum at twenty-four hours. In two subjects absorption was irregular.

TABLE III. SERUM CONCENTRATIONS OF PENICILLIN (UNITS PER CUBIC CENTIMETER) FOLLOWING SINGLE SUBCUTANEOUS INJECTIONS OF 600,000 UNITS (2 C.C.) OF PENICILLIN-BEESWAX-PEANUT OIL MIXTURE

PATIENT	4 HR.	8 HR.	12 HR.	16 HR.	20 HR.	24 HR.	28 HR.
1			0.156	0.039	0	0	
2	2.5	5.0		0.312		0.078	0
3	10.0	10.0		0.078		0.078	0
4			10.0	0.312	0.039	0.039	
5			0.625	0.312	0.312	0.078	
6			10.0	0.156	0.156	0.156	
7			0.625	0.625	0.156	0.156	
8			0.312	0.312	0.312	0.156	
9			0.312	0.156	0.156	0.156	
10			0.156	0	0.156	0.156	
11			20.0	0.625	1.25	0.312	
12			5.0	2.5	1.25	0.312	
13			2.5	2.5	0.625	0.312	
14			0.625	0.312	0.312	0.312	
15			1.25	0.625	0.625	0.312	
16	40.0	5.0		0.625		0.078	0.078
17	2.5	1.25		0.625		0.625	0.156
18	5.0	2.5		0.625		0.312	0.156
19	2.5	0.625		0.625		0.312	0.312
20	2.5	1.25		0.312		0.125	0.312

#### CLINICAL STUDY

Thirty-two patients with various infections have been treated with either sodium or calcium penicillin in wax and oil. In Table IV are shown the results of the treatment of these infections and the dosage schedules employed. The seven patients with gonorrhea had previously failed to respond to doses up to 400,000 units of commercial penicillin in aqueous solution and consequently were treated with a single injection of 600,000 units of penicillin in wax and oil. All had clinical and laboratory evidences of prompt and complete cure which were verified by follow-up examinations two, seven, fourteen, and twenty-one days after treatment. Six patients with gonococcal arthritis were treated with doses of 300,000 to 600,000 units every twelve hours. One patient failed to respond to a course consisting first of 300,000 units and later of 600,000 units given twice a day for a total of fourteen days. No improvement was noted despite the fact that penicillin concentrations of 0.625 unit per cubic centimeter were found in the joint fluid, whereas only 0.039 unit per cubic centimeter were required to inhibit the gonococcus in vitro. There was no response to an additional course of penicillin in isotonic salt solution, 50,000 units intramuscularly every three hours for five days. The patient ultimately recovered coincident with the administration of sulfadiazine.

Two patients with salpingo-oophoritis were treated with penicillin in wax and oil. The causative agent in the case of one patient was the gonococcus; in

TABLE IV. RESULTS OF CLINICAL STUDY OF PENICILLIN IN BEESWAX AND PEANUT OIL

DISEASE	NUMBER OF PATIENTS	PLAN OF THERAPY (UNITS)	RESULTS
Gonorrhea	7	600,000	Recovered
Gonococcal arthritis	6	300,000 to 600,000 once or twice a day for 8 to 10 days	5 Recovered 1 Unimproved
Salpingo-oophoritis	2	300,000 twice a day for 5 days	Recovered
Secondary syphilis	1	300,000 once a day for 16 days	Serologic titer zero 3 months after completion of therapy
Subacute bacterial endocarditis	1	300,000 twice a day for 42 days, then 600,000 once a day for 14 days	No evidence of infection 2 months after completion of therapy
Infections of skin	9	300,000 to 600,000 once or twice a day for 3 to 14 days	Recovered
Erysipelas	1	600,000 once a day for 5 days	Recovered
Otitis media	2	600,000 once a day for 5 to 8 days	Recovered
Tonsillitis and pharyngitis	2	600,000 once a day for 5 days	Recovered
Laryngitis and bronchitis	1	600,000 once a day for 5 days	Unimproved

the other the agent was not identified. Both patients noted marked relief of pain and discomfort within twenty-four hours after treatment was started. The temperature returned to normal within this period of time. Convalescence was uneventful and the drug was discontinued after five days.

One patient with secondary syphilis was given a course of penicillin in wax and oil consisting of 300,000 units once a day for sixteen days for a total of 4,800,000 units. The course of therapy was uneventful, and three months after completion of treatment the patient's serologic titer had fallen to zero.

Penicillin in beeswax and peanut oil was administered to one patient with subacute bacterial endocarditis due to a *Streptococcus viridans*. He made a spectacular response following the initiation of treatment with his preparation in doses of 300,000 units every twelve hours. This was later changed to 600,000 units once a day. The patient made an uneventful recovery following fifty-six days of treatment and has remained free of evidences of infection two months after completion of therapy.

Nine patients with cellulitis and one with erysipelas were treated with doses of 300,000 to 600,000 units once or twice a day for three to ten days with complete resolution of the infection in each case. In one patient the area of cellulitis surrounded a large abscess which was incised and drained. A hemolytic *Staphylococcus aureus* was cultured from the pus. Still another patient developed scarlet fever in addition to the cellulitis, and recovery was uneventful despite the complication.

Two patients with acute catarrhal otitis media and one patient with tonsillitis and pharyngitis caused by a hemolytic streptococcus recovered with the use of this preparation of penicillin in doses of 600,000 units once a day for five

to eight days. The only other therapeutic failure was in a patient with a non-specific laryngitis and bronchitis treated with 600,000 units of penicillin once a day for five days.

#### DISCUSSION

The injection of 300,000 units of penicillin in wax and oil resulted in concentrations of at least 0.156 unit per cubic centimeter of serum twelve hours after administration in seventeen of twenty patients (85 per cent). An additional patient exhibited a concentration of 0.039 unit per cubic centimeter increasing to 90 per cent the incidence of significant concentrations of the antibiotic in the serum at twelve hours. These concentrations of penicillin have been found to be bactericidal for most penicillin-sensitive organisms. Seventy per cent of the patients studied had serum concentrations of 0.039 unit per cubic centimeter, or more, fifteen hours after the injection: 50 per cent exhibited these concentrations at eighteen hours and 25 per cent at twenty-one hours.

Increasing the dose to 600,000 units resulted in serum concentrations of 0.078 unit per cubic centimeter, or more, at twelve hours in thirty-four out of thirty-six patients (94 per cent). Twenty-nine patients (80 per cent) had effective concentrations of the antibiotic in their serum at sixteen hours and nineteen (54 per cent) at twenty hours. One-third of the patients studied were found to have serum concentrations of penicillin of at least 0.078 unit per cubic centimeter twenty-four hours after injection.

The injection of 600,000 units of penicillin in wax and oil subcutaneously resulted in high concentrations for as long as twenty-four hours after injection in all except one of the patients studied.

The concentrations of penicillin in the blood reported by Kirby and associates<sup>3</sup> following the intramuscular injection of 300,000 units of penicillin in beeswax and peanut oil were present for a shorter time than those reported by Romansky and Rittman<sup>2</sup> who used a similar preparation.

Both of these groups of investigators employed a preparation made according to the original formula devised by Romansky. Our results with the modified formula are essentially the same as those obtained by Kirby and associates.<sup>3</sup>

Thirty-two patients with various infections amenable to penicillin were treated with this preparation. The results were as satisfactory as those obtained with penicillin administered in aqueous solution or with penicillin made according to the original formula.<sup>7-12</sup>

Only slight to moderate local pain occurred when the preparation was injected intramuscularly. This discomfort began immediately and usually lasted only a minute or two, very occasionally as long as three or four hours. Most of the patients complained of moderate to marked pain immediately after subcutaneous injection and developed a painful tender nodule which persisted for several days. Eleven days after a subcutaneous injection of 600,000 units of penicillin, one patient developed an area of induration and angioneurotic edema 3 inches in diameter without constitutional symptoms. This subsided within a week on symptomatic measures.



It is obvious that any modification of penicillin which will permit injections to be spaced at twelve- to twenty-four hour intervals will be advantageous, particularly in the case of patients with such diseases as subacute bacterial endocarditis or syphilis who have to be treated for long periods of time. Therapy is simplified in infections which require treatment for several days. This method of treatment is ideal also for patients with gonorrhea since it can be carried out with one injection. The high serum concentrations obtained during the first few hours following injection make it likely that infections caused by organisms which are relatively resistant to penicillin can be treated satisfactorily by proper regulation of the dose and time interval. These advantages have been offset, in the past, by the difficulty inherent in the administration of penicillin in peanut oil and beeswax as prepared by the original Romansky formula. We believe that the modified preparation which we have used eliminates this difficulty in administration, at the same time retaining all the other advantages of the original preparation.

#### SUMMARY AND CONCLUSIONS

1. A new preparation of penicillin incorporated in peanut oil and beeswax has been studied to determine its rate of absorption and its therapeutic efficacy.

2. The intramuscular injection of 1 c.c. containing 300,000 units of penicillin resulted in assayable concentrations of penicillin in the serum for twelve hours in 85 per cent of the patients studied, with measurable concentrations persisting for as long as twenty-one hours in 25 per cent. Following an intramuscular injection of 2 c.c. containing 600,000 units, detectable concentrations were found in 90 per cent of the subjects at twelve hours and in one-third of the patients for twenty-four hours. An injection of 600,000 units subcutaneously resulted in detectable concentrations of penicillin in the blood for twenty-four hours in all but one of the subjects studied.

3. Twenty-seven patients suffering from various infections were treated with doses varying from 300,000 to 600,000 units once or twice per day. The results were similar to those obtained with the use of other preparations of penicillin in beeswax and oil or with equivalent *per diem* doses of penicillin in aqueous solution.

4. The preparation used by us was found to be easier to administer than the preparations made according to the original Romansky formula.

We wish to thank Dr. William W. Zeller, for his assistance in the completion of these studies, and Miss Joan Rowe, for technical assistance.

#### REFERENCES

1. Romansky, M. J., and Rittman, G. E.: Penicillin: Prolonged Action in Beeswax-Peanut Oil, *New England J. Med.* 233: 577, 1945.  
Dept. (no. 81) p. 43, 1944; abstr., *A Method of Prolonging the Action of Penicillin*, *Science* 100: 196, 1944.
2. Romansky, M. J., and Rittman, G. E.: Penicillin Blood Levels for Twenty-Four Hours Following a Single Intramuscular Injection of Calcium Penicillin in Beeswax and Peanut Oil, *New England J. Med.* 233: 577, 1945.
3. Kirby, W. M. M., Leifer, W., Martin, S. P., Rammelkamp, C. H., and Kinsman, J. M.: Intramuscular and Subcutaneous Administration of Penicillin in Beeswax-Peanut Oil, *J. A. M. A.* 129: 940, 1945.

4. Robertson, D. F.: Personal communication.
5. Rammelkamp, C. H.: Method for Determining Concentration of Penicillin in Body Fluids and Exudates, *Proc. Soc. Exper. Biol. & Med.* 51: 95, 1942.
6. Kirby, W. M. M., Martin, S. P., Leifer, W., and Kinsman, J. M.: Maintenance of Therapeutic Blood Concentrations of Penicillin for Twenty-Four Hours Following Single Injections of Penicillin-Beeswax-Peanut Oil Mixtures, *J. LAB. & CLIN. MED.* 31: 313, 1946.
7. Romansky, M. J., Murphy, R. J., and Rittman, G. E.: Single Injection Treatment of Gonorrhea With Penicillin in Beeswax-Peanut Oil; Results in 175 Cases, *J. A. M. A.* 128: 404, 1945.
8. Van Slyke, C. J., and Heller, J. R., Jr.: Treatment of Gonorrhea by a Single Intramuscular Injection of Penicillin-Oil-Beeswax; a Cooperative Study of 1,060 Cases, *Ven. Dis. Inform.* 26: 98, 1945.
9. Graham, W. E., Greenblatt, R. B., and Cannefax, G. R.: Accelerated Methods of Treating Gonorrhea in the Female With Penicillin-Wax-Oil Mixtures, *Ven. Dis. Inform.* 26: 105, 1945.
10. Leifer, W., Martin, S. P., and Kirby, W. M. M.: The Treatment of Gonococcal Urethritis With Single Injections of Penicillin-Beeswax-Peanut Oil Mixtures, *New England J. Med.* 233: 583, 1945.
11. Chinn, B. D., Olansky, S., and Murphy, I. G.: The Treatment of Gonorrhea by a Single Injection of a Penicillin-Oil-Beeswax Mixture, *Med. Ann. District of Columbia* 15: 62, 1946.
12. Geiger, A. J., and Goerner, J. R.: The Treatment of Subacute Bacterial Endocarditis With Penicillin in Peanut Oil and Beeswax, *New England J. Med.* 235: 285, 1946.

# PHARMACODYNAMIC EFFECT IN MAN OF STREPTOMYCIN CONTAINING A HISTAMINE-LIKE FACTOR

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IN A systematic study of the pharmacodynamic effects of streptomycin in animals, it has been demonstrated that the reactions to the pure compound differ from those of preparations which contain impurities.<sup>1</sup> Administration to man of streptomycin containing these impurities has resulted in severe reactions and in two instances death has occurred.<sup>2</sup> Solutions containing one of these contaminants produce a sudden fall in arterial blood pressure, peripheral vasodilatation, and inhibition of water diuresis when injected into rabbits. However, when the solutions are incubated with histaminase prior to injection, these reactions do not occur.<sup>3</sup> Therefore, it has been considered generally that the impurity is histamine. Herein are presented additional data concerning the pharmacodynamic effects of this impurity of streptomycin solutions in man. For convenience, streptomycin solutions containing the histamine-like contaminating substance will be designated H-streptomycin and regular commercial streptomycin will be designated C-streptomycin.\*

## MATERIALS AND METHODS

Young adults who were in the terminal phases of penicillin therapy for primary and secondary syphilis but who were otherwise in good health and gave no personal or familial history of allergic disorder were used as subjects. H-streptomycin was dissolved in sterile distilled water in concentration of 100 mg. per cubic centimeter for intramuscular administration and further diluted to 5 mg. per cubic centimeter in physiologic saline for intravenous use. Control observations were made using C-streptomycin prepared for administration in a similar manner. Benadryl was dissolved in physiologic saline in a concentration of 30 mg. per 50 c.c. for intravenous use.

H-streptomycin was then injected intramuscularly in 100 to 500 mg. doses and intravenously in 50 mg. doses, and observations were made with special attention to peripheral vasodilatation, blood pressure, pulse rate, headache, and palpitation. The effectiveness of benadryl, a potent antihistamine substance, in altering these reactions was next observed by administering a dose of 10 mg. of benadryl intravenously five to seven minutes prior to the intramuscular injection of 500 mg. of H-streptomycin. In other subjects a dose of 10 mg.

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of benadryl was given intravenously one minute after the intravenous injection of 50 mg. of H-streptomycin was begun and when the histamine-like reaction to it was well established. Comparatively small doses of benadryl were used because our supply of the drug was limited.

The effect of H-streptomycin on the gastric secretion was studied in nine subjects by analysis of the volume and free and total acidity of the gastric contents prior to and after the intramuscular injection of 500 mg. of H-streptomycin.

In two asthmatic subjects the effect of 50 mg. of H-streptomycin on the vital capacity was studied by methods previously described.<sup>4, 5</sup> Control observations were made using histamine base and C-streptomycin. The effectiveness of benadryl in preventing the reduction in the vital capacity which followed the injection of H-streptomycin and histamine base was then determined.

#### RESULTS AND COMMENT

Intravenous administration of 50 mg. of H-streptomycin produced a characteristic response in all of twenty patients tested. This consisted of a sensation of facial or generalized warmth, palpitation, throbbing headache, and objective evidence of peripheral vasodilatation by observation of facial suffusion. No notable alteration occurred in the pulse rate, respiration, or blood pressure, as determined by the usual clinical methods. Intramuscular administration of doses up to 500 mg., however, produced these changes in only 48 per cent of twenty-three individuals tested with the largest dose, 500 mg., and the reactions when present were frequently less severe than following intravenous administration of the drug (Table I). In seven subjects, when the intravenous administration of H-streptomycin was initiated and the characteristic response was evident, 10 mg. of benadryl were injected intravenously in the opposite arm. A prompt and marked amelioration of symptoms followed, and rapid blanching of the facial suffusion was noted despite the continued administration of the remainder of the 50 mg. dose of H-streptomycin. A dose of 10 mg. of benadryl was given intravenously to six subjects who previously showed sensitivity to intramuscular H-streptomycin, and five to seven minutes later the intramuscular administration of 500 mg. of H-streptomycin failed to produce the characteristic response.

A stimulating effect of H-streptomycin on the gastric secretion which closely resembles a histamine response is shown in Table II. A significant increase in free acidity and an increased total volume occurred in at least seven of the nine subjects studied.

In two asthmatic subjects it was previously shown that a quantitatively similar decrease in vital capacity followed the intravenous injection of identical amounts of histamine. Therefore, in Subject B. R., after a control injection of 50 mg. of C-streptomycin administered intravenously had produced no notable effect, a dose of 50 mg. of H-streptomycin was given by the same route, and vital capacities were recorded at intervals. The reduction in vital capacity that followed was similar to the reduction produced by the intravenous administration of 0.01 mg. histamine base. The protection afforded

TABLE I. EFFECT OF PARENTERAL ADMINISTRATION OF STREPTOMYCIN CONTAINING HISTAMINE-LIKE FACTOR

NUMBER OF SUBJECTS	DOSE (MG.)	ROUTE OF ADMINISTRATION	HEAD-ACHE	PALPITA-TION	FLUSH	NO REACTION
20	50	I.V.	12	3	19	0
6	100	I.M.	0	0	1	5
21	200	I.M.	2	0	3	17
23	500	I.M.	3	0	9	12

TABLE II. EFFECT OF STREPTOMYCIN CONTAINING HISTAMINE-LIKE FACTOR ON GASTRIC SECRETION

SUBJECT	VOLUME		FREE HYDROCHLORIC ACID	
	BEFORE H-STREPTOMYCIN (C.C.)	AFTER H-STREPTOMYCIN (C.C.)	BEFORE H-STREPTOMYCIN (UNITS)	AFTER H-STREPTOMYCIN (UNITS)
H. T.	36	175	17	97
J. B.	33	75	23	35
G. H.	20	30	0	26
E. R.	15	100	15	60
L. S.	10	36	0	55
J. J.	14	58	0	82
C. N.	50	55	0	90
E. H.	15	40	0	102
R. H.	32	42	0	65

by 30 mg. of benadryl given intravenously against the reduction in vital capacity produced by 50 mg. of H-streptomycin and 0.01 mg. of histamine base likewise was similar. However, when 4 mg. of acetyl- $\beta$ -methylcholine chloride was injected intramuscularly thirty-four minutes after the benadryl was given, a pronounced decrease in vital capacity occurred, and epinephrine was necessary to afford relief from the induced asthmatic-like attack (Fig. 1).

In Subject J. D., after administration of 50 mg. of C-streptomycin had produced no notable effect, 50 mg. of H-streptomycin were injected intravenously, and the vital capacity was recorded at intervals. The reduction in vital capacity that followed was similar to the reaction due to the intravenous injection of 0.02 mg. of histamine base. As in the previous case, 30 mg. of benadryl administered intravenously afforded a similar protection against the reduction in vital capacity due to 50 mg. of H-streptomycin and 0.02 mg. of histamine given by vein. The reduction in vital capacity that followed the intramuscular injection of 1.0 mg. of acetyl- $\beta$ -methylcholine chloride was not affected by the administration of benadryl (Fig. 2). In these two asthmatic subjects the reaction of the tracheobronchial tree to H-streptomycin and to histamine was similar. The protection afforded by 30 mg. of benadryl against H-streptomycin and histamine was likewise similar. The failure of benadryl in the dosage used to protect against the reduction in vital capacity due to acetyl- $\beta$ -methylcholine chloride appears to indicate that the streptomycin contaminant is not related to acetyl- $\beta$ -methylcholine chloride. Since it has been shown previously that under certain circumstances quantitatively similar amounts of bronchoconstriction can be produced by identical amounts of histamine, it may be possible to determine quantitatively the amount of histamine-like contaminant present in streptomycin solutions by this method.

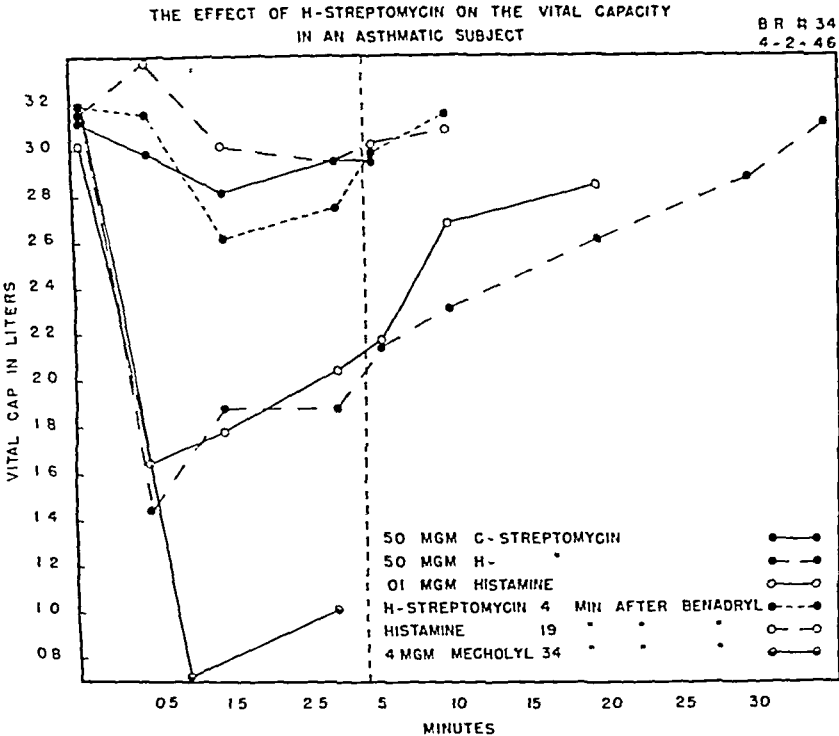


Fig. 1.

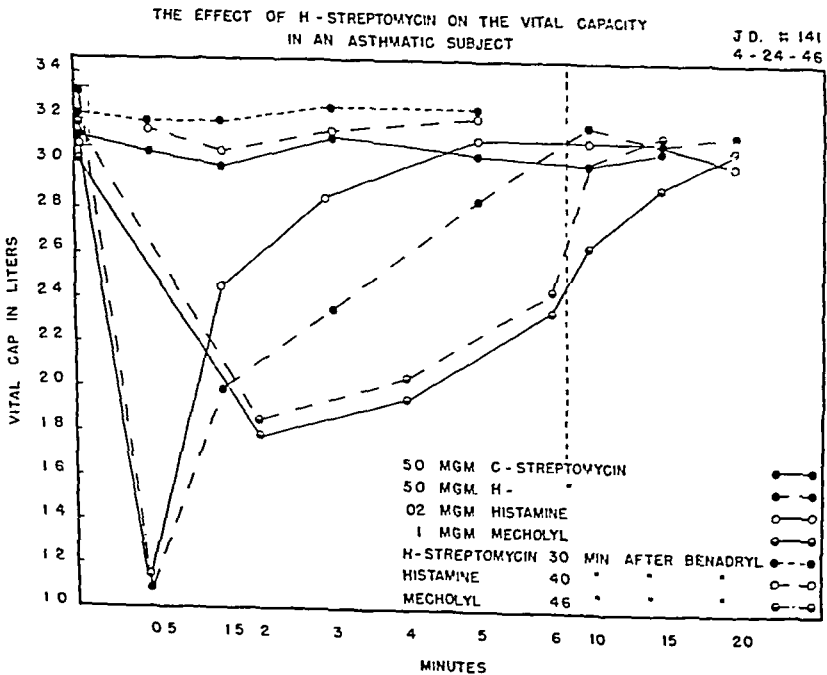


Fig. 2.

## SUMMARY

1. Throbbing headache, palpitation, and peripheral vasodilatation were observed uniformly in twenty young adult subjects following the intravenous administration of 50 mg. of streptomycin containing a histamine-like factor, but a similar histamine-like reaction was observed in only 48 per cent of twenty-three subjects following intramuscular administration of 500 mg. of the same material.

2. Small doses of benadryl given intravenously were effective in ameliorating these reactions to H-streptomycin.

3. In nine fasting young adult subjects, 500 mg. of streptomycin containing a histamine-like factor, administered intramuscularly, produced a significant increase in total volume of gastric secretion and the amount of free acid present.

4. In one asthmatic subject the intravenous administration of 50 mg. of H-streptomycin and 0.01 mg. of histamine base produced a similar degree of reduction in the vital capacity which was almost completely prevented by 30 mg. of benadryl given intravenously. In another subject essentially similar results were achieved when 0.02 mg. of histamine base was administered. Benadryl in the dosage used afforded no notable protection against the reduction in vital capacity due to acetyl- $\beta$ -methyleholine chloride.

5. These studies in man furnish further evidence that one of the contaminating substances of impure solutions of streptomycin is histamine.

## REFERENCES

1. Molitor, H., Graessle, D. E., Kuna, S., Mushett, C. W., and Silber, R. H.: Some Toxicological and Pharmacological Properties of Streptomycin, *J. Pharmacol. & Exper. Therap.* 86: 151-173, 1946.
2. Editorial: Toxicity of Streptomycin, *J. A. M. A.* 131: 745-746, 1946.
3. Editorial: Streptomycin, *J. A. M. A.* 130: 939-940, 1946.
4. Curry, J. J.: The Action of Histamine on the Respiratory Tract in Normal and Asthmatic Subjects, *J. Clin. Investigation* 25: 785-791, 1946.
5. Curry, J. J.: The Effect of Antihistamine Substances and Other Drugs on Histamine Bronchoconstriction in Asthmatic Subjects, *J. Clin. Investigation* 25: 792-799, 1946.

## HISTAMINE ANTAGONISTS

### VI. COMPARATIVE ANTIHISTAMINIC ACTIVITY OF SOME ETHYLENEDIAMINE DRUGS IN THE GUINEA PIG

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RAPID progress in the field of histamine antagonists has resulted in the development of several synthetic compounds which have proved successful to some degree in alleviating certain allergic manifestations in man. All of these substances are chemically and pharmacologically related. Among the first to be introduced in this country was benadryl, a benzhydryl ether of  $\beta$ -dimethylaminoethanol.<sup>1</sup> In France, N' phenyl-N' benzyl-N-dimethylethylenediamine (antergan) has been used clinically since 1942.<sup>2</sup> A search for more potent and less toxic compounds led to the development of two other substances, pyribenzamine<sup>3</sup> and neoantergan.<sup>4</sup> The former differs from antergan only by substitution of the pyridil for the phenyl ring, while neoantergan differs from pyribenzamine only by the addition of a methoxy group on the benzyl ring (Table I). A comparative study of the antihistaminic activity of these four compounds was undertaken, in order to serve as a basis for future studies in anaphylaxis.\*

#### EXPERIMENTAL

The guinea pig responds to intravenous doses of histamine with characteristic bronchospasm and asphyxia difficult to distinguish from that observed in anaphylactic shock. The effect is rapid in onset, and death from fatal doses occurs within a few moments. Post-mortem examination reveals the characteristic pale emphysematous lungs in each instance. Unlike anaphylaxis, non-fatal histamine shock does not confer immunity to subsequent injections of histamine. In the present experiments the histamine was injected into the penile veins of adult male guinea pigs. The intracardiac route was avoided because of the possibility of fatalities from the direct action of histamine on the coronary circulation or other cardiac tissues.

From preliminary experiments<sup>5</sup> we determined that 0.4 mg. per kilogram of histamine,<sup>†</sup> calculated in terms of the base, killed all control animals. Shock occurred in all guinea pigs from 0.3 mg. per kilogram but terminated fatally in only 50 per cent. All animals survived a dose of 0.1 mg. per kilogram of histamine. In order to determine as objectively as possible the degree of antihistaminic activity of each of these compounds, the amount of histamine neces-

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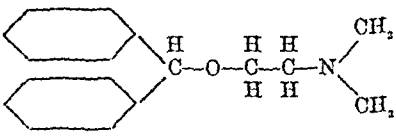
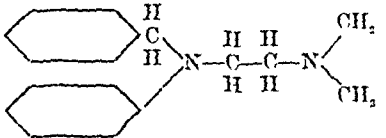
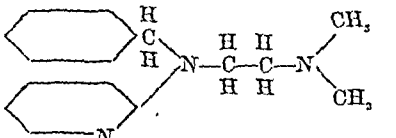
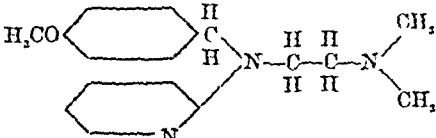
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<sup>1</sup>Benadryl was supplied by Parke, Davis & Company, Detroit, Mich.; pyribenzamine by Ciba Pharmaceutical Products, Inc., Summit, N. J.; antergan and neoantergan by Rhône-Poulenc, Paris, France.

<sup>†</sup>Histamine phosphate was furnished by Burroughs Wellcome & Co. (U. S. A.) Inc., New York, N. Y.



TABLE 1

 <p style="text-align: center;">Benadryl</p>	 <p style="text-align: center;">Antergan</p>
 <p style="text-align: center;">Pyribenzamine</p>	 <p style="text-align: center;">Neoantergan</p>

sary to produce 100 per cent mortality in a group of guinea pigs receiving a standard dose of the drug was determined. In each instance the standard protective dose was 3 mg. per kilogram and was administered intraperitoneally fifteen minutes prior to the intravenous injection of histamine. It was previously determined that none of the solutions produced fatal toxic symptoms on intraperitoneal injection in doses below 50 mg. per kilogram. Animals surviving histamine shock were not used again for at least forty-eight hours.

#### RESULTS (TABLES II AND III)

In those animals protected with 3 mg. per kilogram of benadryl, the dose fatal to all controls (0.4 mg. per kilogram of intravenous histamine) produced only a 29 per cent mortality. Two milligrams per kilogram were necessary to kill all benadryl-protected animals. Antergan gave approximately the same amount of protection, 2.4 mg. per kilogram of histamine being required for the 100 per cent fatal dose in animals receiving this drug. Equivalent doses of pyribenzamine afforded more protection against histamine than either benadryl or antergan. The animals receiving pyribenzamine exhibited little evidence of shock from doses of histamine as high as 2.0 mg. per kilogram. The majority recovered from histamine shock produced by 10 mg. per kilogram, while a dose of 15.0 mg. per kilogram was found fatal for all guinea pigs in this group. Neoantergan, which is only slightly different in chemical structure from pyribenzamine, conferred even greater protection on some animals. While 10 mg. per kilogram of histamine produced approximately the same mortality in this group as in the pyribenzamine-protected guinea pigs, more than half of the neoantergan animals were able to survive doses of 25.0 mg. per kilogram. Fifty milligrams per kilogram of histamine were found to be the 100 per cent fatal dose in this group. On the basis of the number of lethal doses of histamine required to produce 100 per cent mortality in animals protected with a standard

TABLE II. PROTECTIVE EFFECT OF BENADRYL AND ANTERGAN

HISTAMINE I.V. [MG. (BASE)/KG.]	CONTROL GROUP (MORTALITY)		GROUP RECEIVING BENADRYL, 3 MG./KG. (MORTALITY)		GROUP RECEIVING ANTERGAN, 3 MG./KG. (MORTALITY)	
	Total deaths		Total deaths		Total deaths	
	Total used (%)		Total used (%)		Total used (%)	
.03 to 0.1	0/3	0				
0.2	2/6	33				
0.3	3/6	50	0/5	0		
0.4	10/10	100	2/7	29	0/10	0
0.8 to 1.6			2/6	33	6/10	60
2.0			6/6	100	4/5	80
2.4					9/9	100

TABLE III. PROTECTIVE EFFECT OF PYRIBENZAMINE AND NEOANTERGAN

HISTAMINE I.V. [MG. (BASE)/KG.]	CONTROL GROUP (MORTALITY)		GROUP RECEIVING PYRIBENZAMINE, 3 MG./KG. (MORTALITY)		GROUP RECEIVING NEOANTERGAN, 3 MG./KG. (MORTALITY)	
	Total deaths		Total deaths		Total deaths	
	Total used (%)		Total used (%)		Total used (%)	
.03 to 0.1	0/3	0				
0.2	2/6	33				
0.3	3/6	50				
0.4	10/10	100	0/6	0	0/5	0
0.18 to 1.6			0/5	0		
2.0			0/4	0	0/6	0
2.4 to 4.8			1/6	16		
5.0			2/6	33	0/10	0
5.2 to 6.4			1/5	25		
6.8 to 9.0			1/5	25		
10.0			2/5	40	9/24	37
11.0 to 13.0			5/8	62		
15.0			10/10	100	9/19	47
20.0					4/9	44
25.0					5/11	45
30.0					7/10	70
40.0					8/10	80
50.0					10/10	100

TABLE IV. ANTIHISTAMINIC ACTIVITY

3 MG./KG. OF PROTECTIVE DRUG	NUMBER OF LETHAL DOSES OF HISTAMINE NECESSARY TO PRODUCE 100% MORTALITY
Benadryl	5
Antergan	6
Pyribenzamine	37
Neoantergan	125

dose of each drug, pyribenzamine would thus appear to be approximately 6 to 7 times more active than either benadryl or antergan, while neoantergan would possess almost 3.5 times the activity of pyribenzamine (Table IV).

## DISCUSSION

The protective effect of these agents against the lethal effects of histamine is striking and indicates a great degree of specific antagonism. Various investigators have reported that these compounds also exert marked anti-

anaphylactic activity.<sup>2, 3, 5-7</sup> This action together with the beneficial effects observed in certain allergic disorders is in accord with the theory that histamine plays an important role in anaphylaxis and allergy. In our own experiments we also found these drugs capable of modifying anaphylaxis in the guinea pig. Preliminary studies did not show a marked difference in the antianaphylactic activity of these compounds comparable to the variation exhibited in histamine shock. Further experiments are being carried out in order to clarify, if possible, this apparent discrepancy.

#### SUMMARY

A comparison was made of four antihistamine substances, benadryl, pyribenzamine, antergan, and neoantergan. All compounds afforded some protection to guinea pigs against the fatal effects of intravenous histamine. Five to six times the LD 100 of histamine was necessary to kill all animals protected with 3 mg. per kilogram of benadryl or antergan. The same dose of pyribenzamine was able to protect some animals against as much as thirty-five lethal doses, while neoantergan protected some animals against as much as three times this amount of histamine.

#### REFERENCES

1. Loew, E. R., Kaiser, M. E., and Moore, V.: Synthetic Benzhydryl Alkamine Ethers Effective in Preventing Fatal Experimental Asthma in Guinea Pigs Exposed to Atomized Histamine, *J. Pharmacol. & Exper. Therap.* 83: 120, 1945.
2. Halpern, B. N.: Experimental Study of Synthetic Antihistaminic Substances: Chemotherapeutic Trials in Allergic States, *J. de méd. de Lyon* 23: 409, 1942.
3. Mayer, R. L., Huttner, C. P., and Scholz, C. R.: Antihistamine and Antianaphylactic Activity of Some  $\alpha$ -pyridino-ethylenediamines, *Science* 102: 93, 1945.
4. Bovet, D., Horelois, R., and Walthert, F.: Antihistamine Properties of N-p-methoxybenzyl-N-dimethylaminoethyl  $\alpha$  amino-pyridine, *Compt. rend. Soc. de biol.* 138: 99, 1944.
5. Friedlaender, S., Feinberg, S. M., and Feinberg, A. R.: Histamine Antagonists. V. Comparative Study of Benadryl and Pyribenzamine in Histamine and Anaphylactic Shock, *Proc. Soc. Exper. Biol. & Med.* 62: 65, 1946.
6. Loew, E. R., and Kaiser, M. E.: Alleviation of Anaphylactic Shock in Guinea Pigs With Synthetic Benzhydryl Alkamine Ethers, *Proc. Soc. Exper. Biol. & Med.* 58: 235, 1945.
7. Wells, J. A., Morris, H. C., and Dragstedt, C. A.: Modification of Anaphylaxis by Benadryl, *Proc. Soc. Exper. Biol. & Med.* 61: 104, 1946.

## UNUSUAL INSTANCES OF AURICULAR FLUTTER

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THE finding of either 1:1 or persistent 3:1 A-V conduction in auricular flutter is so rare that their occurrence merits attention. Two case histories, each illustrating one of these mechanisms electrocardiographically, are presented.

### CASE REPORTS

CASE 1.—The first case is that of a 42-year-old white man with a bronchogenic carcinoma and extensive metastases which caused almost complete obstruction of the esophagus. A gastrostomy was performed to relieve the effects of compression. The patient became progressively weaker during his hospital stay. On the evening of the forty-ninth postoperative day, the heart rate became rapid (160 beats per minute) and irregular with a pulse deficit. The rate continued to increase until it was too rapid to be accurately counted. The patient's body became covered by a cold and clammy perspiration, the skin developed a cadaveric hue, and loud bubbling râles could be heard throughout the chest. He lost consciousness and the blood pressure could not be obtained. Oxygen therapy was immediately instituted and an electrocardiogram was taken (Fig. 1). This showed a ventricular rate of 296 beats per minute. Deep pressure applied over the right and left carotid sinuses had no effect on the ventricular rate. About three hours after the onset of the rapid heart rate, 0.48 Gm. of quinidine was given intramuscularly with massage and heat over the injection site to aid in absorption. Ten minutes later, one of the house physicians in attendance noted that the heart rate counted at the apex was 148 beats per minute and regular, and "flutter waves" at twice this rate were visible in the neck veins. The patient regained consciousness and responded to questioning. The slower ventricular rate persisted until the patient's death, which occurred several hours later. The slowing of the heart rate so soon after the intramuscular injection of the quinidine naturally raises doubt that it was due to this drug. Unfortunately, an electrocardiogram demonstrating the change in the heart rate was not obtained, but because the slower rate was exactly one-half that of the faster one and because of the visible auricular waves in the neck veins at a rate of 296, it was felt that the mechanism had converted from auricular flutter with 1:1 conduction to one with 2:1 conduction.

*Discussion.*—Some authors<sup>1-3</sup> have referred to several of the difficulties which may be encountered in distinguishing auricular flutter from the paroxysmal tachycardias. However, the gradual onset of the arrhythmia, the extremely rapid and regular ventricular rate (296 beats per minute), the absence of electrical quiescence during the auricular cycles, the normal configuration of the QRS complexes, and the clinical observation of the halving of the ventricular rate from 296 to 148 beats per minute with flutter waves visible in the neck veins established the diagnosis of an auricular flutter with 1:1 conduction in this case.

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The occurrence of transmission of every impulse to the ventricles is extremely rare in auricular flutter. In 1933, Bedell<sup>1</sup> was able to find only twenty-four such cases reported in adults. This is explained by the normally long absolute refractory phase in the A-V node which interferes with the transmission of the frequently recurring auricular impulses, which in auricular

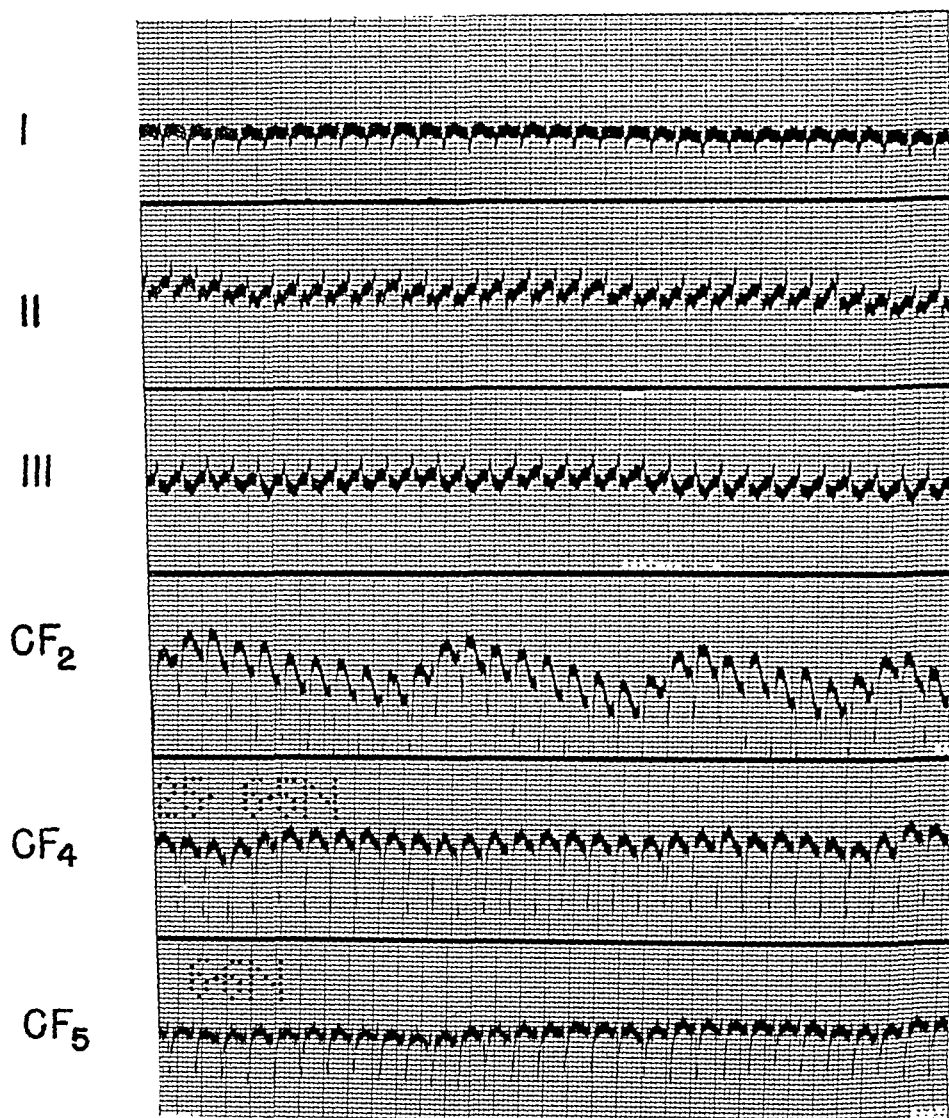


Fig. 1.

flutter have a rate usually between 250 and 400 beats per minute. Only when some circumstance abbreviates the duration of the refractory phase of the A-V node can every impulse be transmitted. Abbreviation of the refractory phase of the A-V node may occur as a result of injury or during sympathetic

stimulation (excitement, adrenalin). Usually this occurs<sup>6</sup> when the flutter rate is slower than 250 beats per minute. Such slowing of the auricular flutter rate is best exemplified by the exhibition of quinidine, whereby the auricular rate may even be slowed to below 200 impulses per minute.

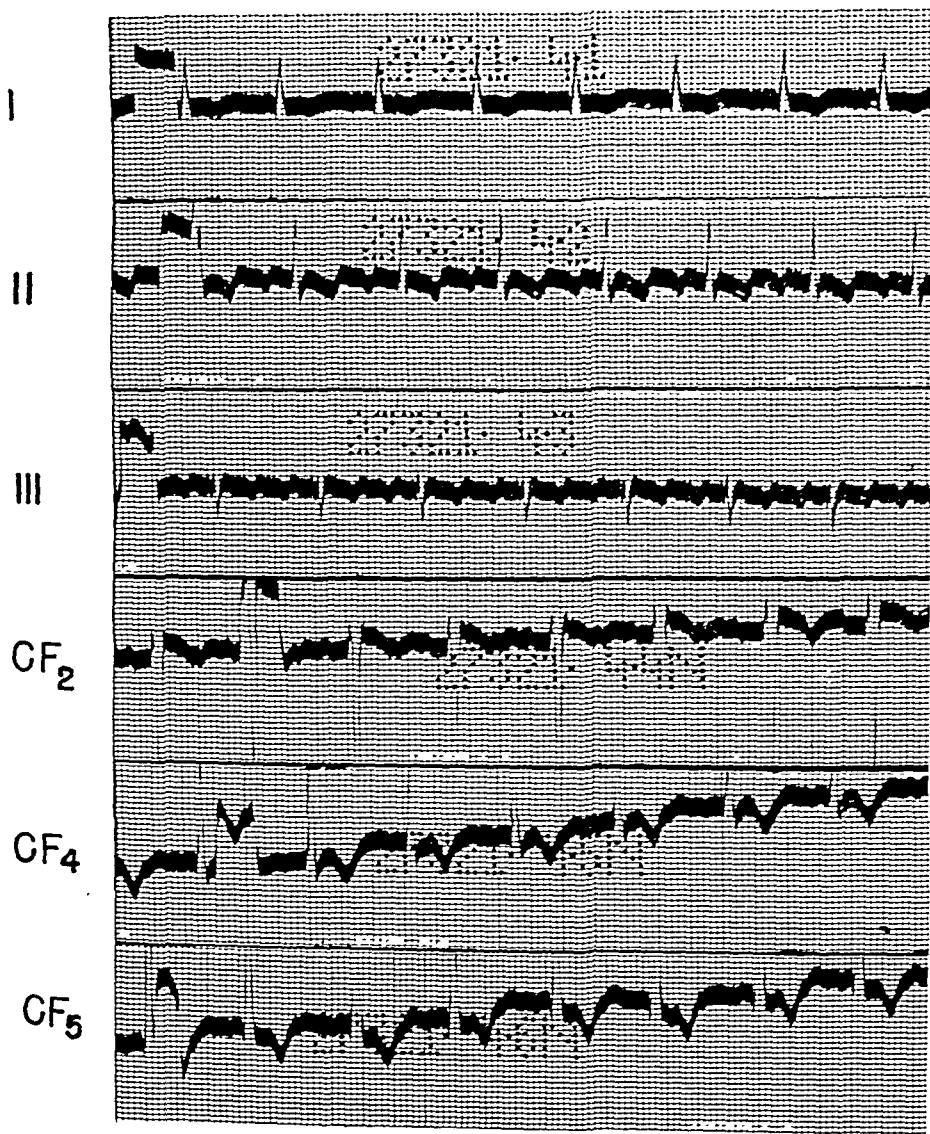


Fig. 2.

The other unusual feature in this case is the fact that 1:1 conduction occurred with an auricular rate of almost 300 making the ventricular rate itself rapid. Such rapid ventricular rates are rare. In 1937, a survey of the literature by Lyon<sup>7</sup> revealed seventeen reported cases with a ventricular rate of over 280 beats per minute. Of these seventeen cases twelve were accompanied by graphic evidence, and of these latter only four (including a 14-

year-old boy) were in adults. Edeiken,<sup>8</sup> in 1943, reported the case of a 46-year-old woman in whom, twenty-two days after an acute myocardial infarction, paroxysms of tachycardia (either supraventricular or 1:1 auricular flutter) occurred with ventricular rates of 310 and 303 beats per minute. Ours is therefore another instance of ventricular tachycardia over 280 which was due to 1:1 auricular flutter.

CASE 2.—This 52-year-old colored woman entered the hospital because of orthopnea, dyspnea on exertion, and swelling of the ankles for the six months prior to admission. An electrocardiogram revealed auricular flutter with a 2:1 A-V conduction. With the institution of therapy, the patient improved clinically. Other electrocardiograms showed persistent flutter with 2:1 and 4:1 A-V conduction and occasional ventricular premature systoles, the auricular rate being about 300 beats per minute. The fourth record (Fig. 2) revealed the very rare phenomenon of auricular flutter with a regular ventricular rate and a persistent 3:1 A-V conduction. Several days after this last tracing, oral quinidine, 0.2 Gm. every two hours and a total dosage of 2.8 Gm., was given following an initial test dose. The auricular flutter persisted, so after a rest period of three days quinidine therapy was again instituted. Within a period of thirty hours the patient received a total of 6 Gm. of quinidine which was divided into three separate courses. Careful check was made for evidence of cinchonism; frequent cardioscopic examinations were made, and an electrocardiogram was taken between each course. Fig. 3A shows the conversion of the flutter mechanism to a sinus rhythm with bigeminy (ventricular premature systoles). The bigeminy may have been due, in part at least, to digitalis (0.2 mg. digitaline nativele daily) which the patient received throughout the period of quinidine administration. All medication was discontinued after the bigeminal rhythm was found, and an electrocardiogram taken forty-eight hours later showed a sinus rhythm with only an occasional ventricular premature systole (Fig. 3B).

Discussion.—Occasional cycles with 3:1 conduction in cases of auricular flutter with an irregular ventricular rate are not uncommon. However, the occurrence in auricular flutter of a persistent 3:1 conduction is extremely rare.<sup>6, 9</sup> Thus, this case is the first one in which this phenomenon has been found in over 30,000 electrocardiograms taken at the Michael Reese Hospital. The very regular, saw-toothed flutter waves, occurring at a rate of 270 cycles per minute, are best seen in Leads II and III of Fig. 2. Closer inspection will disclose their presence in the other leads as well. We are aware of only three such previous cases on record.<sup>10-12</sup> It has been postulated that 2:1 A-V conduction in auricular flutter is due to a focal interference which prevents every second auricular impulse from stimulating the ventricles; 4:1 A-V conduction would occur if a second block existed which would allow only one-half of the impulse that had penetrated the first block from reaching the ventricles. This would readily explain the relative frequency of 2:1 A-V conduction and the less common occurrence of 4:1 A-V conduction. However, this theory cannot account for the existence of a persistent 3:1 A-V conduction. The presence, even on occasion, of persistent 3:1 conduction indicates either that the hypothesis previously stated is erroneous or that a single region of interference may become so protracted as to allow the penetration of only one out of every three beats.

Another interesting feature in this case is the establishment of a bigeminal rhythm after the flutter mechanism was broken (Fig. 3A). Finally, in Fig. 3B

is illustrated the quinidine effect on the electrocardiogram with marked T-wave inversions (except in Lead III) and the prolongation of electrical systole (0.40 second; normal, 0.29 to 0.37 second).

A

B

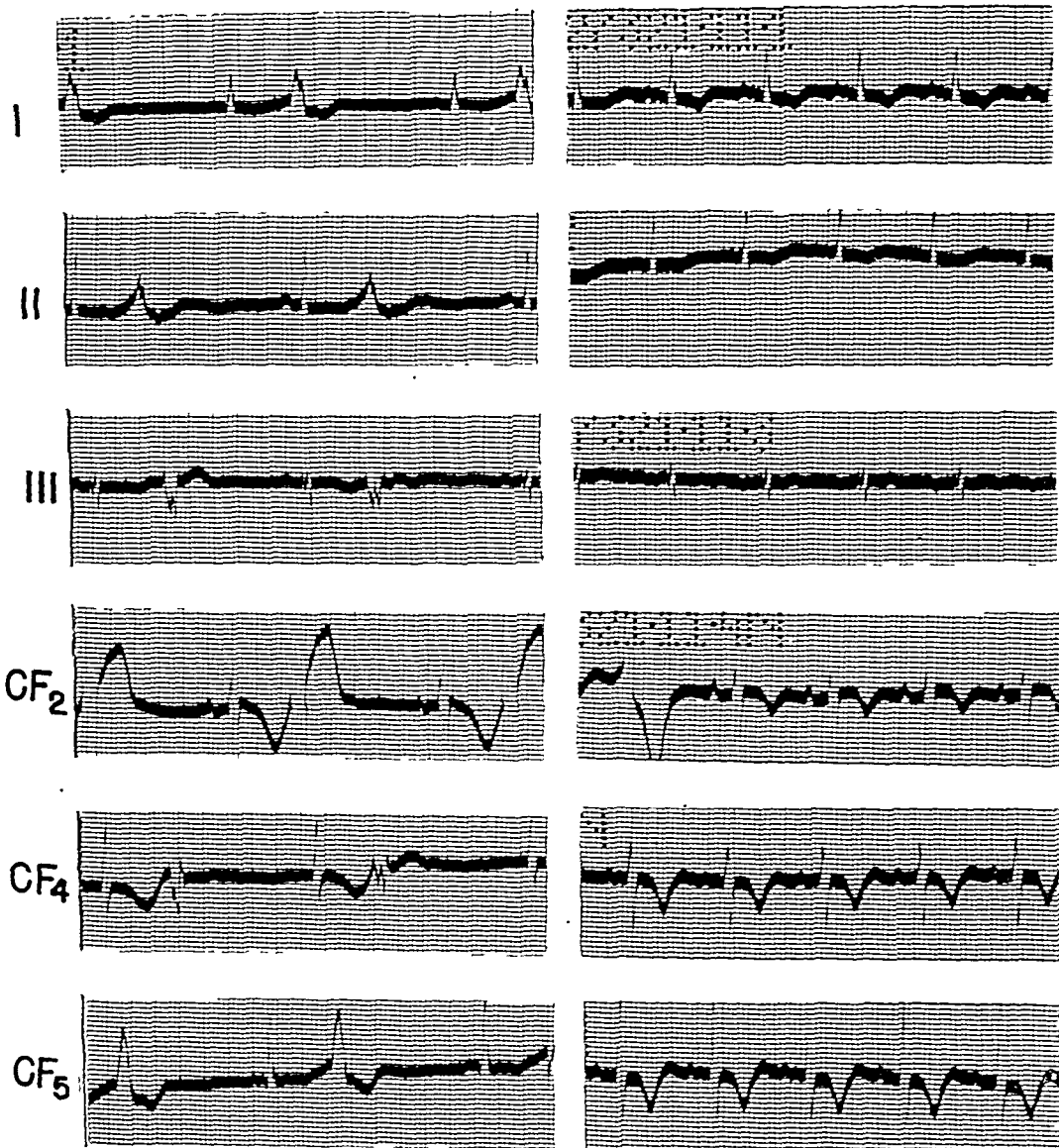


Fig. 3.

## SUMMARY

1. A very rare instance of auricular flutter with a 1:1 conduction and a ventricular rate of 296 beats per minute is presented.



2. An even rarer phenomenon is shown, namely, a case of auricular flutter with a persistent 3:1 A-V conduction.

## REFERENCES

1. Decherd, G. M., Jr., Herrmann, G. R., and Schwab, E. H.: Paroxysmal Supraventricular Tachycardia With A-V Block, *Am. Heart J.* 26: 446, 1943.
2. Evans, W.: The Unity of Paroxysmal Tachycardia and Auricular Flutter, *Brit. Heart J.* 6: 221, 1944.
3. Barker, P. S., Wilson, F. N., Johnston, F. D., and Wishart, S. W.: Auricular Paroxysmal Tachycardia With Auriculoventricular Block, *Am. Heart J.* 25: 765, 1943.
4. Bedell, C. C.: Auricular Flutter With 1:1 Response, *Bull. Johns Hopkins Hosp.* 52: 225, 1933.
5. Pardee, H. E. B.: *Clinical Aspects of the Electrocardiogram*, ed. 4, New York, 1941, Paul B. Hoeber, Inc., p. 219.
6. Katz, L. N.: *Electrocardiography*, ed. 2, Philadelphia, 1946, Lea & Febiger, p. 666.
7. Lyon, J. A.: Excessively Rapid Heart Rates. Report of a Case With Autopsy, *J. A. M. A.* 108: 1393, 1937.
8. Edeiken, J.: Extreme Tachycardia, With Report of Nonfatal Paroxysms Following Myocardial Infarction, *Am. J. M. Sc.* 205: 52, 1943.
9. Ashman, R., and Hull, E.: *Essentials of Electrocardiography*, ed. 1, New York, 1937, The Macmillan Company, p. 186.
10. Graybiel, A., and White, P. D.: *Electrocardiography in Practice*, ed. 1, Philadelphia, 1941, W. B. Saunders Company, p. 280.
11. Sigler, L. H.: *The Electrocardiogram*, New York, 1944, Grune & Stratton, Inc., p. 131.
12. Wenckebach, K. F., and Winterberg, H.: *Die unregelmässige Herztätigkeit*, Vienna, 1927, Wilhelm Engelmann, p. 170.

# EFFECT OF OXYGEN ON MALARIA

## AN IN VIVO STUDY IN DUCKS

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**P**ATHOLOGIC studies on a child dying from a *Plasmodium falciparum* infection,<sup>1</sup> on monkeys dying from *Plasmodium knowlsi* infection,<sup>2</sup> and on ducks dying from *Plasmodium lophurae* infection<sup>3</sup> showed lesions that were similar to those occurring in shock. It was suggested that they resulted from anoxemia. This anoxemia resulted from the anemia produced by the rapid destruction of red blood cells by the plasmodia. Subsequent studies have shown that the blood of ducks with a severe malarial infection at the time of death may have only 15 to 20 per cent of the normal blood oxygen carrying capacity.<sup>4</sup> Anoxemia in malaria is again manifest when ducks with malaria are placed in a decompression chamber in that they succumb before the non-infected birds. The length of survival in these malaria-infected ducks, when in the presence of decreasing amounts of oxygen, may be correlated with the degree of parasitemia.

The clinical observations of Kean and associates<sup>5, 6</sup> support the role that shock may play in acute malarial infections in man. They presented six cases in support of the opinion that medical shock may develop in patients with algid malaria. In three of their cases there was "hemoconcentration, evidence of an increase in the hematocrits, red blood cell count, and hemoglobin. The blood pressure was lowered and there were other signs of vasomotor collapse. . . . The final evidence for shock in these cases was the response to replacement therapy. Plasma was used in three cases, whole blood transfusion in one, oxygen in two, 'Eschatin' in two, intravenous fluids in five, and epinephrine in five."

Ducks with a severe malarial infection show clinical improvement immediately when given a transfusion of duck blood.<sup>7</sup> The red blood cells replace those destroyed by the parasites; thereby more oxygen can be transferred to the tissue. A study has been made to determine the effect of increasing the amount of oxygen available to ducks to see the effect upon the course of the disease. The results of this study are reported at this time.

### METHODS AND MATERIAL

White Pekin ducks 2 to 4 weeks of age were used. Blood from donor birds infected with *P. lophurae* was used to infect the ducks. In each experiment a group of birds from the same hatching were inoculated intravenously with blood from the same donor bird.

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The course of the infection was followed by counting the number of parasitized cells per 500 red blood cells. Blood films were stained with a combination of Giemsa's and Wright's stains.

Total red blood cell counts were made on some of the ducks. Haymen's fluid was used as the diluent. Hematoerit readings were obtained by centrifuging 10.0 c.c. of heparinized blood for thirty minutes in Wintrobe tubes. Liquemiu was the anticoagulant and was used in a dilution of 1 to 9.

The oxygen chamber was 60 by 32 by 24 inches in size. It was made from galvanized iron supported by an angle iron frame. The bottom had a 2 inch drop from one end to the other. The top of the chamber was made of glass. The door was located at one end of the chamber. A wire meshed floor was placed 3 inches from the bottom. A 2 inch pipe was put in the bottom of the chamber at its lowest point. Water was run continuously into a container used for the ducks to drink from, while the overflow ran over the bottom of the chamber and out into a floor drain. Two small holes were made through the top of the chamber. A thermometer was kept in one and samples of oxygen were removed through the other.

A tank of oxygen was placed adjacent to one end of the chamber. A regulator was used to control the flow of oxygen. The rubber tube carrying the oxygen entered the chamber through a hole in the top. The concentration of oxygen within the chamber was determined frequently with an oxylizer.\* The concentration of oxygen varied during the experiments; usually it was kept at a concentration of 50 to 60 per cent during the first few days of the disease and then increased to 80 to 90 per cent. In some experiments the birds were kept continuously within the oxygen chamber for days, while in others they were removed daily for short intervals to obtain blood for the tests. Samples for hematoerit determination were obtained by cardiac puncture, and the birds were discarded thereafter. Blood for cell counts and films were obtained by puncturing a vessel in either the leg or web of the foot.

#### EXPERIMENTAL

*Variations in Erythrocyte Count of Normal Ducks Kept in Oxygen Chamber.*—Six young ducks were kept in the oxygen chamber for two weeks with a 50 to 70 per cent concentration of oxygen. The average red blood cell count in these birds is shown in Fig. 1. During the first five days there was a diminution in the total number of red cells, after which the number rapidly returned to normal levels. The erythrocyte count remained within the range of normal during the twelve subsequent days during which the birds were kept in the oxygen chamber. There was no variation in the red cell count for six days after the ducks were removed from the oxygen chamber.

*Effect of Oxygen on Parasitemia in Ducks Infected With P. lophurae.*—Twenty ducks 15 days of age were inoculated with malaria and ten were put into the chamber with a concentration of 50 per cent oxygen. The parasitemia was followed in five birds in the oxygen chamber and in five of the group kept in the duck room. The average degree of parasitemia in these two groups of ducks is shown in Fig. 2. The parasitemia is the same in the two groups until the fourth day following which time there is an increase in the number of parasites in the oxygen-treated group when compared with those kept in the duck room. By the sixth day of the disease, eight of the ten ducks with malaria kept in the duck room were dead, while only five of the ten ducks

\*The Ohio Chemical & Mfg. Company, Cleveland, Ohio.

with malaria kept in the oxygen chamber were dead. The concentration of oxygen was increased to approximately 75 to 80 per cent on the morning of the sixth day as shown in Fig. 2. The remaining five ducks in the oxygen chamber survived until the morning of the seventh day. These five ducks died within seven minutes following the time they were removed from the oxygen chamber.

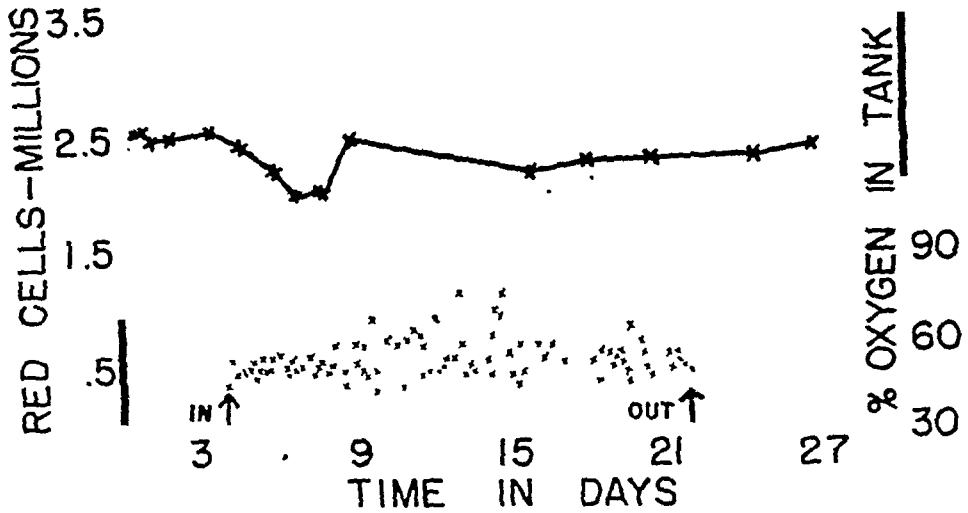


Fig. 1.—Changes which occur in the number of red cells in normal ducks kept in an oxygen chamber.

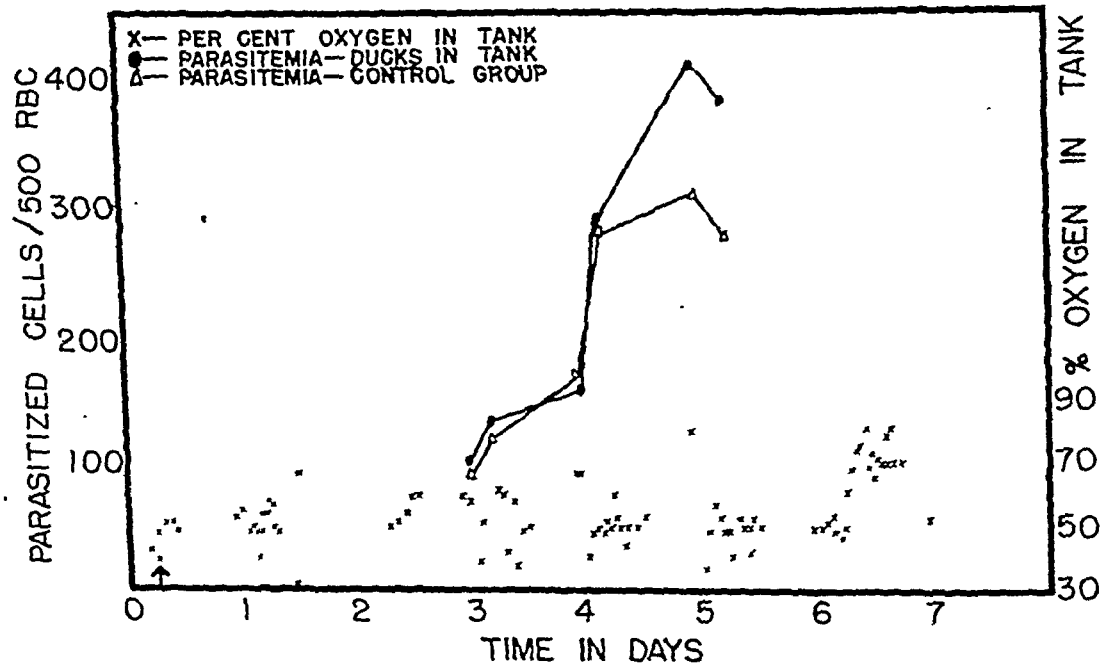


Fig. 2.—The average degree of parasitemia in five ducks kept in the oxygen chamber and of five ducks kept in normal atmosphere. The concentration of oxygen is indicated on the graph.

TABLE I

EXPERIMENTAL DAY	OXYGEN CHAMBER	CONTROLS KEPT IN DUCK ROOM
1	4.1	3.0
2	9.4	8.0
3	16.7	15.6
5 (A.M.)	285.3	127.9
5 (P.M.)	290.0	126.0
6 (A.M.)	325.0	158.0
6 (P.M.)	300.0	137.9
7 (A.M.)	129.0	59.3
7 (P.M.)	95.0	26.0

The effect of oxygen on the parasitemia in ducks was determined in a second experiment in which forty-one birds 16 days of age were used. Twenty of these were put into the oxygen chamber on the fourth day following inoculation. The concentration of oxygen was kept at approximately 50 per cent for twenty-four hours, after which time it was increased to 85 to 90 per cent for the following two days when the experiment was discontinued. The average parasitemia of twenty of these birds was 286 parasitized cells per 500 red blood cells on the fourth day, at the time that the twenty ducks were put into the oxygen chamber. The average parasitemia of ten of these ducks in

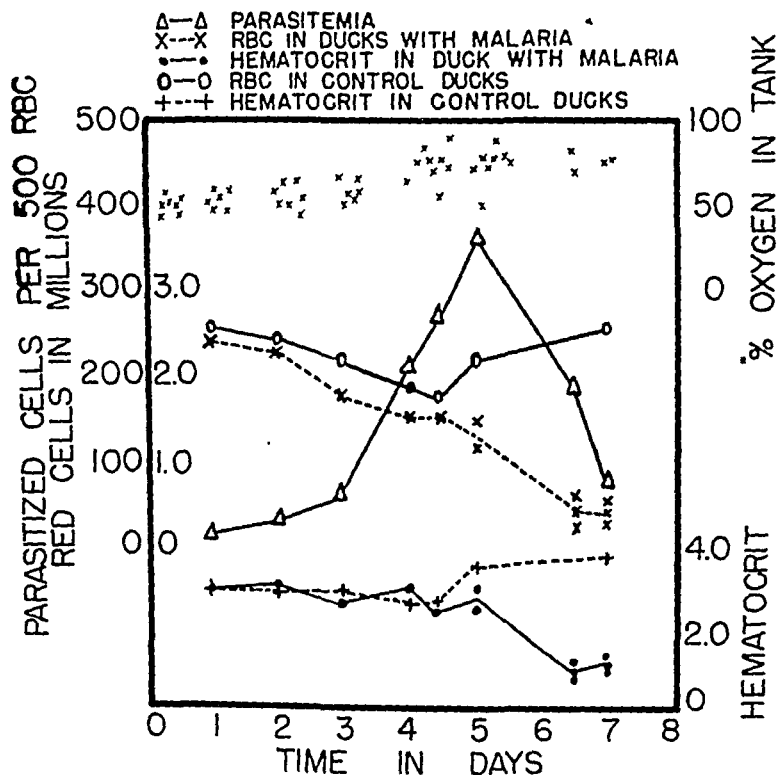


Fig. 3.—The red cell counts, the hematocrit readings, and the parasitemia as observed in ducks infected with *P. lophurae* kept in the oxygen chamber. Two malaria-infected ducks were used to establish each point, except on the sixth day when five birds were used. One normal duck was used to establish the daily values for the controls. A progressive anemia occurred in the malaria-infected birds, while a temporary anemia occurred in the noninfected birds kept under the same environmental conditions.

the oxygen chamber after eighteen hours was 420 parasitized cells per 500 red blood cells, while the average of twenty control ducks was 340 cells. At 9:45 A.M. on the sixth day, eighteen ducks were dead from the group of twenty-one given malaria and kept in the duck room, while only eleven birds were dead from the group of twenty given malaria and put in the oxygen chamber. One of the group removed from the oxygen chamber died about five minutes later.

In a third experiment, twenty ducks 18 days of age were inoculated and ten were put immediately into the chamber with a 50 per cent concentration of oxygen and kept there until the fifth day, at which time the concentration of oxygen was increased to approximately 80 to 85 per cent. The experiment was discontinued on the afternoon of the seventh day. At this time five ducks were dead from the group of ten inoculated with malaria and put in the oxygen chamber, while only one was dead from the group of ten kept as controls in the duck room. The average degrees of parasitemia per 500 erythrocytes in these two groups of ducks are given in Table I.

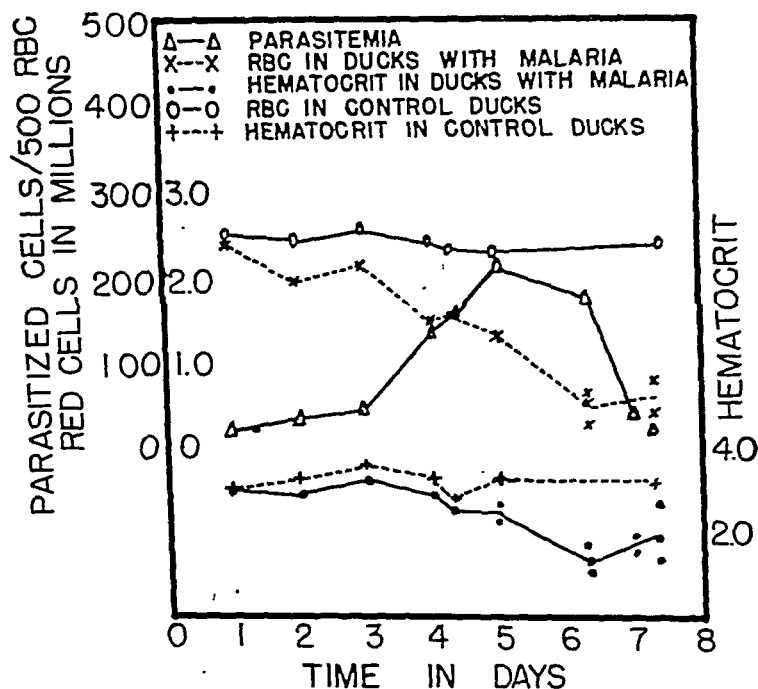


Fig. 4.—The experiment as shown in Fig. 3 was repeated with the ducks kept in the room rather than being put into the oxygen chamber. The parasitemia is much less in this group of birds than in those kept in the oxygen chamber. The anemia and the hematocrit readings are similar to those shown in Fig. 3.

The degree of parasitemia is much greater in the group kept in the oxygen chamber than it is in the control group. The peak of parasitemia apparently was reached at approximately the same time in the two groups. It is not surprising to find a larger number of birds dying in the oxygen-treated group than in the control group, since the parasitemia was much higher in the oxygen-treated group of ducks.

The red blood cell count, the hematocrit readings, and the degree of parasitemia were followed in two groups of ducks, one of which was kept within the oxygen chamber for the entire experiment and the second in batteries in the duck room. A total of forty-four ducks 16 days of age were used. The previously mentioned observations were made upon a different bird each day after which it was discarded. The concentration of oxygen within the chamber, the red blood cell count, the hematocrit reading, and the degree of parasitemia are shown in Fig. 3. The results of similar observations on the control group are shown in Fig. 4. The peak of parasitemia was reached on the fifth day in both groups of ducks; however, the parasitemia was much higher in those kept in the oxygen chamber than it was in the control group. A progressive decrease occurred in the number of red cells and in the hematocrit readings in all ducks regardless of whether they were in the control group or in the oxygen chamber. The red blood cell counts and the hematocrit readings of nonmalarial birds kept in the oxygen chamber with the infected birds decreased during the first four days following which time they rapidly increased to within the range of normal (Fig. 3). Normal ducks used for the controls did not show any significant variation in the red cell count and hematocrit reading during a corresponding interval as shown in Fig. 4.

#### CLINICAL EFFECT OF OXYGEN ON DUCKS WITH MALARIA

Normal ducks kept in the chamber in the presence of 50 to 60 per cent concentration of oxygen showed no clinical symptoms different from those of normal birds kept in the batteries in the duck room. Ducks inoculated with *P. lophurae* and put immediately in the oxygen chamber progressively became pale and weak and ultimately succumbed in a manner identical with that of the ducks with malaria kept in the duck room. However, ducks with a high degree of parasitemia, an anemia, weakness, and a rapid respiratory rate, when put into the chamber with a 50 to 60 per cent concentration of oxygen, immediately showed an increase in activity, and the respirations decreased in frequency and apparently became deeper. The ducks looked markedly improved almost immediately.

There is little difference in the time that death occurs in groups of ducks put into the oxygen chamber immediately following inoculation when compared with the controls kept in the batteries in the duck room. There is, however, a definite increase in the length of survival of malaria-infected birds put into the oxygen chamber on the fourth day of the disease as compared with a similar group kept only in the duck room as shown in Fig. 5.

In this experiment, twenty-five ducks 16 days of age were inoculated with malaria. On the fourth day at 10:00 A.M. the average parasitemia in ten of these ducks was 298 parasitized cells per 500 red blood cells, and in the afternoon it was 304. Ten of the group of twenty-five ducks were put into the oxygen chamber at 4:00 P.M. on the fourth day of the disease. These ducks were carefully watched during the following forty-eight hours to determine the time of death. The oxygen chamber was kept closed during this time. The

time of death and the concentration of oxygen within the chamber are shown in Fig. 5. At 11:30 A.M. on the sixth day, eight of the ten ducks in the oxygen chamber were dead, while twelve of the 15 birds with malaria kept outside of the chamber were dead. Thirty-five minutes following the time of removal of the chamber, one died; the second survived for twenty-four hours after removal. It is obvious from Fig. 5 that ducks with a high degree of parasitemia survive for a much longer time when put into a chamber with a high concentration of oxygen than similarly infected ducks kept at normal atmospheric pressure.

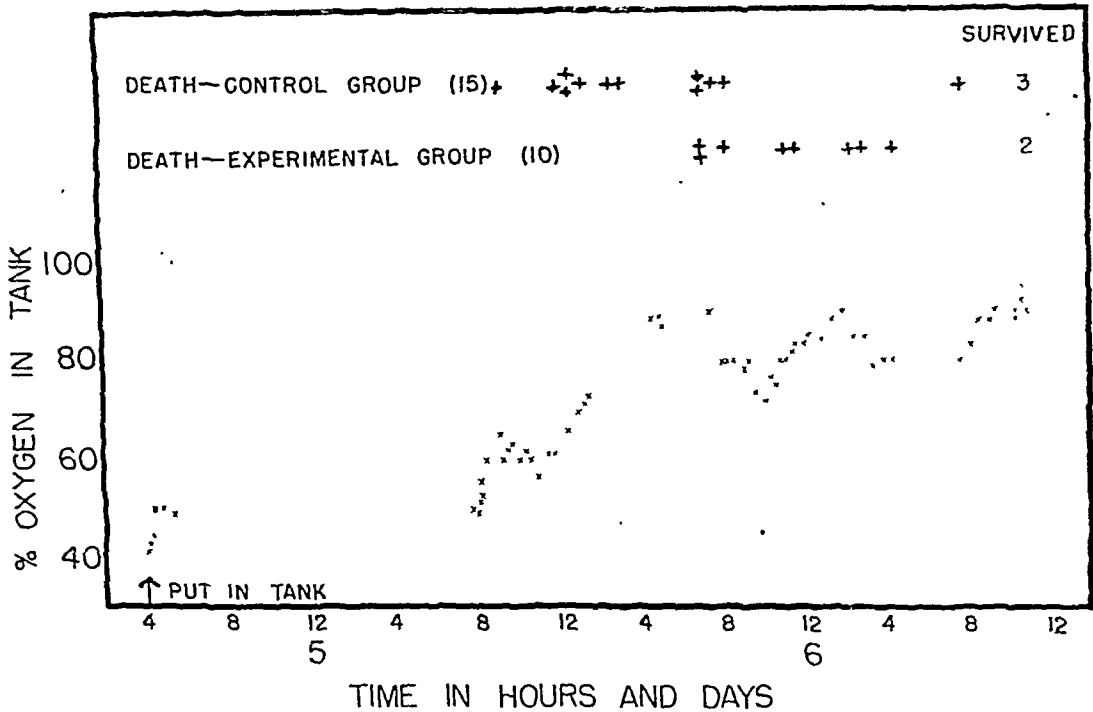


Fig. 5.—The time at which death occurred in the oxygen-treated birds and the controls is indicated here. The concentration of oxygen in the chamber is shown in this figure.

#### DISCUSSION

The experimental observations as made in these studies indicate that oxygen is beneficial to ducks with a severe malarial infection. The increased amount of oxygen apparently helps to compensate for the decrease in the amount of hemoglobin which is present in the highly infected birds. The anemia results from the rapid destruction of red cells by the plasmodia. In *P. falciparum* infections in man the total decrease in the number of red cells may not be so great in some cases; however, the important factor in all types of acute malaria seems to be the rapidity in which the anemia may develop. The fact that ducks with a severe malarial infection show immediate improvement, either following a transfusion with duck red cells or when they are put in the presence of an increased concentration of oxygen, would suggest that clinical sub-



jects with acute malaria and symptoms of shock should be given a transfusion and placed in an oxygen chamber at the same time that a plasmodial drug is given.

The fact that ducks with a *P. lophurae* infection show a higher degree of parasitemia when kept for the entire period of the disease in the presence of an increased concentration of oxygen does not contradict the beneficial effects that may result from the use of oxygen, since it has been shown experimentally that ducks with a severe infection when placed in the oxygen chamber show immediate clinical improvement. An interval of a few hours is important in the treatment of acute malaria, since it requires such a time for the effects of the plasmodial drugs to manifest themselves upon the parasites, and during this interval the patient may need supportive therapy for the anoxemia. It of course would be useless to kill the plasmodia and permit the patient to die from medical shock. As far as it is known there are no contradictions to the use of transfusions and oxygen in the treatment of a patient with malaria.

Cullen and Boothby and their associates<sup>6, 9</sup> have given the indications and rationale of oxygen therapy. Cullen and associates have emphasized the fact "that there is a  $5\frac{1}{2}$  to  $7\frac{1}{2}$  per cent increase in metabolism for every degree (Fahrenheit) of elevation in temperature; it would seem logical to provide these individuals with oxygen in excess of that present in the atmosphere."<sup>8</sup> Patients with acute malaria, therefore, would be benefited by oxygen if they had only an increase in their metabolic rate as a result of the fever. Alkalosis present during fever furthermore effects a significant reduction in the arterial oxygen tension even in the presence of a normal arterial oxygen saturation.<sup>8</sup> "The administration of oxygen benefits the patient chiefly by physical methods. That is, an increase in the alveolar concentration increases the partial pressure, thereby increasing the saturation of the blood with a consequent increase in tension in spite of the elevated pH. The break in the vicious cycle of low tension, high pH, and tissue hypoxia may also be effected by the increased amount of oxygen in physical solution in the plasma, which oxygen is readily available to the tissue and not dependent on dissociation of hemoglobin."<sup>8</sup>

Experimental and clinical studies indicate that shock may occur in severe malarial infections.<sup>1, 2, 6</sup> In the treatment of shock from any cause, all efforts should be directed to improve the circulation of blood through the tissue so that oxygen can be delivered at a higher partial pressure. Administration of 100 per cent oxygen is the most direct and specific method to increase the oxygen pressure in the capillaries and tissue fluids.<sup>9</sup> A 100 per cent concentration of oxygen has been administered to patients for forty-eight hours without any evidence of pulmonary irritation.<sup>9</sup>

There is nothing in these experimental studies to indicate that oxygen per se will cure malaria. In fact, these studies show that the parasitemia in *P. lophurae* infection in ducks kept in the presence of a high concentration of oxygen is higher than that which occurs in ducks kept in the presence of the concentration of oxygen as occurs in the normal atmosphere. Experimental studies have indicated that the sudden decrease in the degree of parasitemia which occurs following the peak of parasitemia in *P. lophurae* infec-

tion in ducks may be the result of a lack of oxygen which apparently is essential for the survival and multiplication of these plasmodia in vivo.<sup>10</sup>

The clinical improvement, as observed in ducks with a severe malarial infection when given oxygen, supports the opinion that anoxemia is a significant factor in the mechanism of death in malaria. All of our physiologic and pathologic studies have shown the effects of a lack of oxygen in malaria,<sup>1-4, 11</sup> and now the results from the use of oxygen and the results following transfusions show the therapeutic effects which may be accomplished in the treatment of acute malaria as suggested from these studies in the duck.

#### SUMMARY

Ducks with a severe malarial infection when placed in an oxygen chamber immediately show marked clinical improvement. They survive for ten to eighteen hours longer than similarly infected ducks kept in the duck room. This improvement is attributed to the greater amount of oxygen available for transmission to the anoxic tissues.

The use of oxygen in the treatment of acute malarial infections in man is discussed.

#### REFERENCES

1. Rigdon, R. H.: A Consideration of the Mechanism of Death in Acute Plasmodium Falciparum Infection; Report of a Case, *Am. J. Hyg.* 36: 269, 1942.
2. Rigdon, R. H., and Stratman-Thomas, W. K.: A Study of the Pathological Lesions in *P. knowlsi* Infection in *M. Rhesus* Monkeys, *Am. J. Trop. Med.* 22: 329, 1942.
3. Rigdon, R. H.: A Pathological Study of the Acute Lesions Produced by Plasmodium lophurae in Young White Pekin Ducks, *Am. J. Trop. Med.* 24: 371, 1944.
4. Rostorfer, H. H., and Rigdon, R. H.: Anoxemia in Malaria; an Experimental Study in Ducks, *J. LAB. & CLIN. MED.* 30: 860, 1945.
5. Kean, B. H., and Taylor, C. E.: Medical Shock in the Pathogenesis of Algid Malaria, *Am. J. Trop. Med.* 26: 209, 1946.
6. Kean, B. H., and Smith, J. A.: Death Due to Estivo-Autumnal Malaria; a Résumé of 100 Autopsy Cases 1925-1944, *Am. J. Trop. Med.* 24: 317, 1944.
7. Rigdon, R. H., and Varnadoe, N. B.: Transfusions of Red Cells in Malaria; an Experimental Study in Ducks, *Am. J. Trop. Med.* 25: 409, 1945.
8. Cullen, S. C., Weir, E. F., and Cook, E.: The Rationale of Oxygen Therapy During Fever Therapy, *Anesthesiology* 3: 123, 1942.
9. Boothby, W. M., Mayo, C. W., and Lovelace, W. R.: One Hundred Per Cent Oxygen; Indications for Its Use and Methods of Its Administration, *J. A. M. A.* 113: 477, 1939.
10. Rigdon, R. H.: Consideration of the Mechanism by Which a Rapid Diminution Occurs in the Parasitemia in *P. lophurae* Infection in Ducks. To be published.
11. Rigdon, R. H., and Rostorfer, H. H.: Blood Oxygen in Ducks With Malaria, *Nat. J. Malaria*. To be published.

# DETERMINATION OF NORMAL MEAN CORPUSCULAR WEIGHT (ERYTHROCYTE)

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OUR knowledge in various types of anemia has been put on a much more sound basis since the discovery of methods of determining and interpreting the mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration of red cells. It occurred to me that additional help might be derived from the estimation of the mean corpuscular weight of the erythrocyte. This determination was carried out in eighty-one normal subjects including ten Chinese and seventy-one Filipinos. The determination was performed according to the same principles used for the mean corpuscular volume, employing the Wintrobe tube.

## METHOD AND RESULTS

All the subjects studied were medical students and doctors of normal development and nutritional status. The erythrocyte count, hemoglobin, leucocyte count, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were found to be within normal limits in each instance. The determination of mean corpuscular weight was made from the same sample of blood.

The capacity of several standard Wintrobe tubes was accurately determined by weighing, on an analytic balance, tubes emptied and filled with distilled water. The capacity of each tube used in these measurements was known to the nearest tenth of a milligram. For simplicity in presentation of the data, a uniform capacity of 0.7 c.c. between the 0 and 100 mm. marks is assumed; in the actual calculations the capacity of each individual tube was used. The technique of the determination was as follows.

The weight of a standard Wintrobe tube was carefully measured. The tube was filled with blood exactly to the 100 mm. mark and centrifuged for at least thirty minutes at 3,000 revolutions per minute. After the hematocrit reading had been taken, the supernatant plasma was removed by capillary pipette without disturbing the cellular sediment. In doing so a column of plasma, platelets, and leucocytes, a few millimeters in length, was left on top of the red blood cells. The tube containing the packed red blood cells and a small amount of plasma was then weighed. When the weight of the empty tube was subtracted from this weight, the weight of the cells and the residual plasma was obtained. The weight of 1 c.c. of plasma made from the same blood sample was measured with a separate set of glassware. From the latter the weight of 0.7 c.c. of plasma was calculated. Dividing the weight of the 0.7 c.c. of plasma by 100, we obtained the weight of a 1 mm. column of the plasma in the Wintrobe tube. By assuming that the density of leucocytes and platelets is equal to that of plasma, and by application of a suitable coefficient to the measured length of the plasma remaining in the tube, we could determine

the weight of the residual material. The weight of the blood cells in 1 c.c. of whole blood was derived as follows.

If it is assumed that the tube is of uniform bore, then:

$$\text{Weight of blood cells in 1 c.c. of whole blood in mg.} = \frac{\text{Weight of Wintrobe tube containing the packed cells and residual plasma} - \text{Weight of tube and weight of residual plasma}}{0.7}$$

$$\text{Weight of cells of 1 c.mm. in mg.} = \frac{\text{Weight of blood cells/c.c.}}{1,000}$$

$$\text{Weight of cells of 1 c.m. in } \mu\text{Gm.} = \frac{\text{Weight of cells/cc.}}{1,000} \times 1,000,000,000$$

$$\text{M.C.W.*} = \frac{\frac{\text{Weight of cells/c.c.}}{1,000} \times 1,000,000,000}{\text{R.B.C./c.mm. in million} \times 1,000,000}$$

$$\text{M.C.W.} = \frac{\text{Weight of cells/c.c. in mg.}}{\text{R.B.C./c.mm. in million}} \text{ micromicrograms}$$

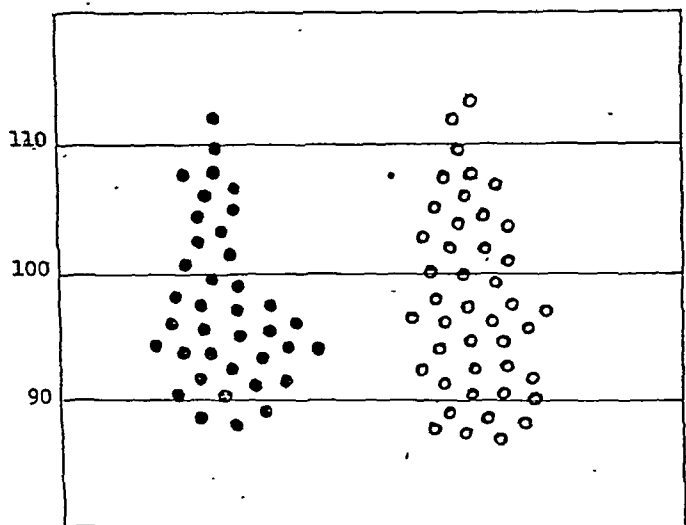


Fig. 1.—Distribution of mean corpuscular weight of thirty-eight men, ●, and forty-three women, ○ (μGm.).

The mean corpuscular weights of the eighty-one subjects are presented in Fig. 1. It is significant that in thirty-four of the thirty-eight men and in thirty-five of the forty-three women, the mean corpuscular weight falls within the range of 90 to 110 micromicrograms. The figures for the entire series calculated according to standard statistical methods are shown in Table I. The general hematologic data of the series are shown in Table II.

TABLE I. MEAN CORPUSCULAR WEIGHT (μGm.) IN EIGHTY-ONE NORMAL SUBJECTS

	NUMBER OF CASES	MEAN $\pm \frac{\sigma}{\sqrt{N}}$	S. D. $\pm \frac{\sigma}{\sqrt{2N}}$
Men	38	96.9 $\pm 1.04$	6.43 $\pm 0.74$
Women	43	96.6 $\pm 1.29$	7.47 $\pm 0.81$
Whole series	81	96.8 $\pm 0.78$	7.02 $\pm 0.55$

\*M. C. W., mean corpuscular weight.

TABLE II. HEMATOLOGIC DATA OF EIGHTY-ONE NORMAL SUBJECTS (TEN CHINESE AND SEVENTY-ONE FILIPINOS)

	PRESENT SERIES		FILIPINO STANDARD		AMERICAN STANDARD	
	MEN (38 CASES)	WOMEN (43 CASES)	MEN	WOMEN	MEN	WOMEN
R.B.C. (million)	5.26 (4.24-5.91)	4.74 (4.19-5.35)	5.1 (4.2-5.5)	4.5 (4.0-5.0)	(4.8-6.5)	(4.3-5.5)
W.B.C.	8,000 (5,600-10,500)	7,600 (5,600-9,500)	7,600 (6,300-8,800) *	7,500 (6,700-8,500)	(5,000-10,000)	(5,000-10,000)
Hemoglobin (Gm.)	14.2 (11.8-15.8)	13.3 (11.8-14.7)	14 (12-17)	12 (11-14)	(14.5-18.5)	(12.5-15.0)
Hematocrit	46 (40-50)	41 (37-46)	41 (40-50)	37 (37-45)	(40-50)	(37-45)
Mean corpuscular volume	88.2 (76-95)	87.9 (78-96)	84 (76-95)	83 (75-95)	(75-95)	
Mean corpuscular hemoglobin	27.0 (26-29)	27.4 (26-30)	27 (22-34)	27 (22-34)	(26.5-31.5)	
Mean corpuscular hemoglobin concentration	30.5 (28-34)	31.4 (29-35)	27 (23-32)	27 (23-32)	(33-39)	
Mean corpuscular weight	96.9	96.6				

## DISCUSSION

The author has no knowledge that the mean corpuscular weight of erythrocytes has been studied previously. Without clinical application in pathologic conditions it is difficult to evaluate the significance of this determination. However, it is reasonable to assume that the mean corpuscular weight should be increased (hyperponderal) in macrocytic hyperchromic anemia and should be reduced (hypoponderal) in microcytic hypochromic anemia. In such conditions the determination of the mean corpuscular weight might serve as a collaborative method in differential diagnosis.

With known methods of study changes of the erythrocyte, other than changes of size, shape, and hemoglobin content, cannot be determined. In this respect the mean corpuscular weight might give additional information, not only in the study of anemia but also in other conditions causing disturbance of fluid and salt balance. However, this is something for future study to prove.

## SUMMARY

1. A method of determining the mean corpuscular weight of erythrocytes is presented.

2. The mean corpuscular weight was found to be  $96.9 \mu\text{Gm.}$  (S. D. = 6.43) for thirty-eight normal men and  $96.6 \mu\text{Gm.}$  (S. D. = 7.74) for forty-three women, with an average of  $96.8 \mu\text{Gm.}$  (S. D. = 7.02) for both sexes.

3. The application and significance of this determination are to be ascertained by future study in pathologic conditions.

This study was made in the laboratories of the Cancer Institute of the University of the Philippines, Manila, when the author was stranded there by the Pacific War in 1944. The author wishes to express her gratitude to the members of the laboratories for this privilege and also to Dr. Eugene Stransky of the Philippine General Hospital.

## REFERENCES

1. Wintrobe, M. M., and Miller, M. W.: The Volume and Hemoglobin Content of the Red Blood Corpuscle, *Am. J. M. Sc.* 177: 513, 1929.
2. Wintrobe, M. M.: A Simple and Accurate Hematocrit, *J. LAB. & CLIN. MED.* 15: 287, 1929.
3. Heller, V. G., and Paul, H.: Changes in Cell Volume Produced by Varying Concentrations of Different Anticoagulants, *J. LAB. & CLIN. MED.* 19: 777, 1934.
4. Price-Jones, C., Vaughan, J. M., and Goddard, H. M.: Hematological Standards of Healthy Persons, *J. Path. & Bact.* 40: 503, 1935.
5. Walters, O. S.: Normal Erythrocyte, Hemoglobin and Packed Cell Volume Standards in Young Men, *J. LAB. & CLIN. MED.* 19: 815, 1934.

## THE BONE MARROW OF NORMAL CATS

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### INTRODUCTION

SINCE Arinkin's<sup>1</sup> original description of sternal marrow aspiration in human beings, the method has been applied to dogs by Alexandrov.<sup>2</sup> Meyer and Bloom<sup>3</sup> studied normal canine bone marrow obtained by aspiration of the iliac crest, utilizing the technique of Ilo and associates<sup>4</sup> who were concerned with investigations in leishmaniasis. Lawrence and co-workers<sup>5</sup> refer to the normal bone marrow of the cat in their paper on feline agranulocytosis. These investigators studied histopathologic sections of bones which were fixed in Zenker's fluid, embedded, and cut. They also examined films made from marrow taken from the humerus and femur which were macerated in human serum and stained with Wright's fluid. This method permitted little opportunity for repeated examinations, and the maximal number of observations made on any animal was three. The majority of animals were sacrificed before they were studied. The procedure to be introduced overcomes this disadvantage because of its simplicity of performance and the availability of the same animal for innumerable punctures. In presenting the data we also compare our observations on the peripheral blood with those of other investigators.

### MATERIAL AND METHODS

Studies were made on fifteen apparently normal laboratory cats, both male and female, of unknown age and ranging in weight from 1.69 to 3.43 kilograms each. In the cat the innominate bones articulate with the sacrum and extend caudoventrally, turning medially to unite in the midline to form the symphysis. The ilium extends from the sacrum to the acetabulum. It is contracted in its mid portion and broader at the periphery. The proximal end is thickened, forming the crest, and runs roughly parallel to the vertebral column.

The technique for marrow puncture is as follows: The animal is strapped to the table with dorsum presenting and extremities extended. The hair over the sacral area is clipped and the skin sterilized with any suitable antiseptic. Local anesthesia is not required. The anterior-superior border of the iliac crest is outlined with the fingers of one hand. A sterile 18 to 19 gauge spinal tap needle with straight stylet, about 1½ inches long, is inserted through skin and muscle close to the iliac crest. Upon reaching the periosteum the needle is simultaneously rotated and pushed with a dextro-levo boring movement until the medullary cavity is entered. The marrow cavity has been entered when the needle seems firmly imbedded in the bone and only rarely is a sudden "give" experienced. The stylet is removed, a dry 20 c.c. syringe is attached to the needle, and about 0.1 or 0.2 c.c. of marrow is aspirated. The needle, together with the attached syringe, is then removed from the animal and the drop expelled on a clean glass slide. From the drop white cell and megakaryocyte counts are made with the same technique as for counting peripheral blood. Thin films are made

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on several clean glass slides and stained with Wright's stain. Five hundred nucleated cells are counted and the percentages estimated for the differential count from the stained films, utilizing the classification of Vogel and associates.<sup>6</sup> In Fig. 1 is shown the aspiration needle in the iliac crest.

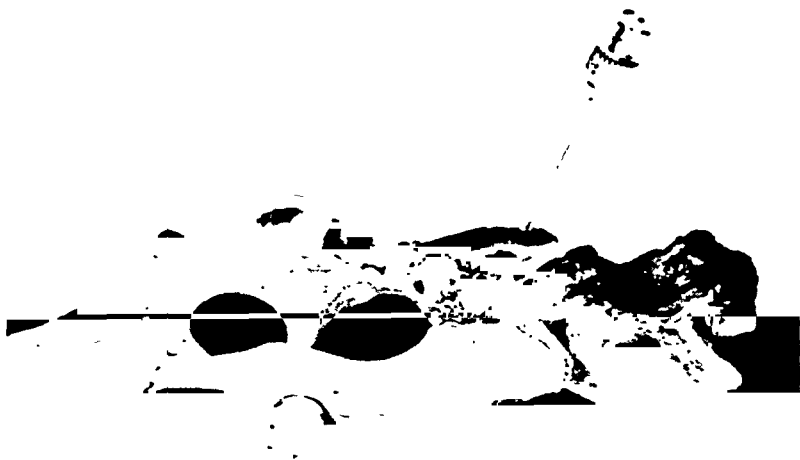


Fig. 1.—Aspirating needle in position in the iliac crest.

#### OBSERVATIONS

A comparison of the data obtained in the peripheral blood (Table I) with those of other observers (Table II) shows a similarity of results which indicates that the animals utilized in the present study were apparently normal. The only exception is Cat 672 in which the leucocyte count was 40,500 per cubic millimeter. The animal appeared to be well.

The bone marrow from the iliac crest (Table III.) shows an increased number of nucleated cells and megakaryocytes as compared with that of human beings and dogs (Table V). The myeloid series constitutes 69.9 per cent of the nucleated cells which are distributed mainly among the segmented (22.5 per cent) and nonsegmented (30.5 per cent) neutrophils. The metamyelocytes constitute only about 13 per cent of the total number. This increased cellularity of the bone marrow with maturation of cells of the myeloid series is apparently specific for the cat, as it is not observed in either the dog or man. The erythroid and lymphatic cells constitute 20.1 and 9.0 per cent, respectively, which are about the same percentages as seen in canine and human bone marrow. As was noted in dogs, many of the cells of the myeloid series contain no granules, but identification of the cells is not difficult because of the ease in identifying nuclear structures. A comparison of the bone marrow data with that of Lawrence and co-workers (Table IV) is unsatisfactory since these workers included in their tabulation 26.3 per cent degenerated cells. Their erythroid and lymphoid percentages, however, compare favorably with those obtained by us.



TABLE I. PERIPHERAL BLOOD OF NORMAL CARS

CAT	R.B.C.	HEMOGLOBIN	W.B.C.	BANDS	SEGMENTED				LYMPHOCYTES	MONOCYTES	EOSINOPHILS	
					NEUTRO- PHILES	PHILES	LYMPHOCYTES	MONOCYTES				
1	10.5	6.75	15,600	2.0	56.0	39.0	0.0	3.0	1.0			
669	12.2	7.9	10,000	2.5	40.0	44.0	4.0	9.5	--			
670	12.5	7.20	14,150	2.0	72.5	17.5	3.5	5.5	--			
671	10.5	7.95	21,300	0.5	68.0	24.5	2.5	4.5	--			
672	11.0	6.0	40,500	2.0	65.0	23.5	5.0	4.5	--			
673	12.5	6.9	21,300	3.0	74.5	18.0	3.0	1.5	1.0			
674	13.8	7.3	16,550	0.5	55.0	39.5	1.5	3.5	--			
675	12.0	6.6	19,250	1.0	70.0	22.0	2.0	5.0	--			
676	12.0	9.0	25,300	1.0	70.0	24.75	1.5	2.75	--			
677	11.5	7.85	22,800	2.5	68.0	25.0	1.0	3.5	--			
678	14.8	8.25	14,400	1.0	65.0	27.5	1.5	4.5	0.5			
679	13.8	7.3	20,000	1.0	73.5	15.5	3.0	7.0	--			
680	12.0	6.5	18,400	1.5	81.5	13.5	1.5	2.0	--			
681	9.5	6.8	28,500	3.0	72.0	18.5	2.5	4.0	0.5			
682	10.8	7.05	16,400	2.0	69.5	22.0	2.0	4.5	3.5			
Average	11.96	7.22	20,300	1.7	66.7	21.9	2.3	4.1	0.5			
Range	9.5 to 14.8	6.0 to 9.0	10,000 to 40,500	0.5 to 3.0	40 to 81.5	13.5 to 44.0	0.0 to 5.0	1.5 to 9.5	0.0 to 1.0			

TABLE II. COMPARISON OF DATA ON BLOOD VALUES OF CATS BY DIFFERENT AUTHORS<sup>7</sup>

TABLE II. COMPARISON OF DATA ON BLOOD VALUES OF CATS BY DIFFERENT AUTHORS								
AUTHOR	R.B.C.	HEMOGLOBIN	LEUCOCYTES (1,000)	DIFFERENTIAL LEUCOCYTE COUNT (PER CENT)				
				P.M.N.	EOSINOPHILES	BASOPHILES	LYMPHOCYTES	MONOCYTES
Bethe	8.71	--	7.20 to 15.15	--	--	0	34.5	4.9
Busch and von Bergen	6.61	--	7.22 to 19.00	51.1	6.5	0	20.0 to 35.0	2.5 to 3.5
Hübner	9.7 to 11.1	12.5 to 15.6	5.15 to 15.20	34.0 to 89.8	1.7 to 4.2	0.1 to 0.4	33.5	2.1
Arndt	7.97	--	--	59.4	5.0	0	25.0	1.5
Kleinberger and Chul	7.74	15.1	10.40 to 29.00	68.5	5.0	--	--	--
Drastich	8.57	11.3	--	--	5.0	0	32.0	6.0
Scarborough	7.10	10.4	13.00 to 14.00	57.0	4.0	0.1	33.4	2.5
Wirth	8.00	10.4	10.00 to 15.00	60.0	1.0	1	8.1 to 45.2	1.2 to 3.4
Vault	7.85	12.8	9.22 to 24.05	41.6 to 84.4	0.8 to 18.0	0	33.0	0.8
Dandberg	7.23	10.19	8.30 to 35.25	59.3	6.9	0	21.9	2.3
Present authors	7.22	11.96	20.3	68.4	4.4	0	--	--

TABLE III. BONE MARROW OF NORMAL CATS

CAT	TOTAL MARROW COUNT × 1,000	TOTAL MEGAKARYOCYTES PER CHAMBER	MYELOBLASTS	MYELOCYTES	MYELOCYTES	MYELOCYTES	BANDS	SIRI-MOUNTED PHILIPPS	EOSINOPHILIC MYELOCYTES	SIRI-MOUNTED EOSINOPHILS	LYMPHOCYTES	PLASMA CELLS	RETICULO-ENDOTHELIAL CELLS	MITOBLASTS	ERYTHROBLASTS	NORMOBLASTS
1	128	--	0.5	6.0	3.25	26.25	22.5	22.5	2.0	1.0	13.5	0.0	0.0	2.0	2.0	25.0
469	222	13	1.25	5.0	7.0	21.0	20.25	20.25	2.0	1.5	5.0	1.0	1.5	0.25	4.0	31.75
670	213	22	0.75	6.75	6.0	30.75	25.0	25.0	1.0	0.25	6.5	0.5	0.5	1.25	21.25	18.75
671	204	39	1.0	6.75	8.75	23.75	26.25	26.25	1.0	3.0	7.25	1.0	1.0	0.5	1.75	15.0
672	230	7	0.25	1.75	9.0	35.25	34.5	34.5	1.0	2.25	6.25	1.5	1.5	0.75	0.75	11.6
673	282	27	1.0	6.6	12.4	38.8	18.8	18.8	0.8	1.0	1.8	0.6	0.6	0.2	0.2	25.6
674	200	17	1.2	1.6	4.4	30.0	12.8	12.8	1.0	3.2	15.8	1.2	1.2	1.2	0.4	28.6
675	147	22	0.6	2.4	1.0	19.6	28.4	28.4	1.6	2.6	11.6	1.6	0.2	0.2	0.2	12.4
676	282	30	0.6	1.8	1.0	33.0	28.8	28.8	2.0	0.8	11.6	1.6	0.2	0.2	1.2	14.6
677	290	10	1.0	8.4	14.6	38.8	10.6	10.6	0.6	0.8	8.4	1.4	0.2	0.4	0.0	5.6
678	173	13	2.0	6.0	18.2	40.4	11.4	11.4	0.4	1.0	9.6	1.4	0.2	0.2	0.8	18.0
679	300	10	0.4	4.6	6.1	30.6	22.0	22.0	2.6	3.2	11.0	0.2	0.2	0.5	0.5	14.3
680	134	4	0.5	1.5	3.4	32.4	32.4	32.4	1.0	1.5	10.7	1.0	0.2	0.5	0.0	10.4
681	193	10	0.8	7.4	9.6	42.8	18.4	18.4	1.0	2.8	5.0	0.8	0.2	0.8	0.0	10.4
682	151	6	1.0	5.75	7.75	15.5	23.5	23.5	0.5	0.75	8.75	0.25	0.25	3.5	3.5	32.5
Average	209.9	20	0.82	5.22	7.96	30.59	22.52	22.52	1.1	1.71	6.65	0.75	0.35	1.24	1.24	18.52

TABLE IV. COMPOSITION OF DATA ON BONE MARROW OF CATS BY DIFFERENT OBSERVERS

BONE MARROW	LAWRENCE AND CO-WORKERS <sup>5</sup>	PRESENT AUTHORS
Number of animals	13	15
Total count		209,900/c.mm.
Megakaryocytes		20/c.mm.
Myeloblasts	1.3	0.82
Promyelocytes	7.6	
Myelocytes		
Neutrophilic	4.6	5.22
Eosinophilic		1.1
Metamyelocytes	9.8	7.96
Nonsegmented		
Neutrophiles	13.4	30.59
Segmented		
Neutrophiles	5.3	22.52
Eosinophiles	1.5	1.71
Lymphocytes	7.8	9.05
Plasma cells	0.3	0.75
Reticulum cells		0.02
Megaloblasts	0.7	0.35
Erythroblasts	6.3	1.24
Normoblasts	8.3	18.52
Unclassified	6.2	
Disintegrated	26.3	
Erythroid-myeloid ratio	0.35	0.29

TABLE V. COMPARISON OF BONE MARROW IN MAN, DOGS, AND CATS

BONE MARROW	MAN <sup>6</sup>	DOG <sup>3</sup>	CAT
Count x 1,000	118.75	144	209.9
Megakaryocytes		41.2	20
Myeloblasts	1.6	0.58	0.82
Myelocytes, agranulocytic	0.1		
Myelocytes			
Neutrophilic	21.5	3.76	5.22
Eosinophilic	0.77	0.26	1.1
Metamyelocytes			7.96
Nonsegmented			
Neutrophiles	30.2	23.50	30.59
Eosinophiles	0.39	0.12	
Segmented			
Neutrophiles	34.0	18.50	22.52
Eosinophiles	0.94	1.56	1.71
Basophiles	0.07	0.02	
Heterophiles		0.02	
Lymphocytes	8.6	9.80	9.05
Pathologic lymphocytes		0.04	
Monocytes		1.20	
Monoblasts		0.14	
Plasma cells		0.82	0.75
Reticulum cells	0.25	0.54	0.02
Hematogones	3.1	0.44	
Megakaryocytes	0.2		
Megaloblasts	0.14	1.02	0.35
Erythroblasts	7.1	2.50	1.24
Normoblasts	22.6	53.18	18.52

## SUMMARY

1. A method is presented describing the technique for obtaining bone marrow from the iliac crest of the cat by aspiration.
2. Data are given showing the cellular constituents in the peripheral blood and bone marrow of normal cats.
3. A comparison of the bone marrow findings in cats with those of dogs and man is made, utilizing the method of aspiration in all three groups.

## REFERENCES

1. Arinkin, M.: Methodology of Examining Bone Marrow in Live Patients, With Hematopoietic Diseases, *Vestnik Khir.* (No. 30) 10: 57, 1927.
2. Alexandrov, A. P.: Die Morphologie des Sternumpunctates von Hundem, *Folia haemat.* 41: 428, 1928.
3. Meyer, L. M., and Bloom, F.: Bone Marrow of Normal Dogs, *Am. J. M. Sc.* 206: 637, 1943.
4. Ho, E. A., Chu, H. S., and Yuan, I. C.: Ilium Puncture; A Simple Method for Obtaining Bone Marrow From Dog, *Chinese M. J.* 58: 679, 1940.
5. Lawrence, J. S., Syverton, J. T., Shaw, J. S., and Smith, F. P.: Infectious Feline Agranulocytosis, *Am. J. Path.* 16: 333, 1940.
6. Vogel, P., Erf, L. A., and Rosenthal, N.: Hematological Observations on Bone Marrow Obtained by Sternal Puncture, *Am. J. Clin. Path.* 7: 436, 1937.
7. Landsberg, J. W.: The Blood Picture of Normal Cats, *Folia haemat.* 64: 169, 1940.

## LABORATORY METHODS

### REACTIONS OF VARIOUS PARASITIC ORGANISMS IN TISSUES TO THE BAUER, FEULGEN, GRAM, AND GRAM-WEIGERT METHODS

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IN THE course of some experimental comparisons of the Best carmine method and the Bauer<sup>1</sup> reaction for glycogen, some cat muscle containing Sarcosporidia chanced to be included in the test material. With the Bauer method on low magnification the Sarcosporidia appeared red purple in a pale gray blue background. On higher magnification they were seen to contain numerous red purple granules in their cytoplasm around the nucleus in the equatorial area of fusiform parasites. After salivary digestion according to Bensley<sup>2</sup> the Sarcosporidia were still red by the Bauer method, while muscle glycogen no longer stained. With Best's carmine method Sarcosporidia do not take the red stain.

This experience led to more extended examination of various tissue parasites by the Bauer method. In most instances it was necessary to use paraffin sections of old material fixed for other purposes in 10 per cent formalin, Orth's fluid, Helly's fluid, or other fixatives.

#### METHODS

The technique of the Bauer reaction and the composition of the Schiff reagent used for both the Bauer and Feulgen tests were substantially as recently reported.<sup>3</sup> By this method glycogen is stained a deep purplish red. The reaction may be prevented by salivary<sup>2</sup> or by enzyme digestion.<sup>3</sup> Cartilage matrix is usually colored a deep reddish purple, contrasting fairly well with the lighter purplish red glycogen in the cartilage cells. Cartilage matrix sometimes remains unstained. The staining of cartilage matrix is not affected by prior salivary, diastase, or ptyalin digestion.

Bauer<sup>1</sup> stated that mucus did not react to this method; however, as Bensley<sup>2</sup> notes and as we found, in mammalian material, mucus is usually stained purplish pink to light purplish red. This staining reaction persists after salivary, ptyalin, or diastase digestion. Mucous stains well by this method after aqueous formalin or Orth fixations.

Mast cell granules were not demonstrated by either the Bauer or the Feulgen techniques in human, cat, or rat material in which they were readily demonstrable with our azure-eosin technique.

When enzyme digestion tests were used, the digestion was performed on deparaffinized, hydrated sections without collodion treatment and after digestion sections were dehydrated and collodionized as previously mentioned before testing with the Bauer technique. The technique is as recently reported by us.<sup>3</sup> For comparison the Feulgen, Gram, and Weigert methods were used as well on most of the material.

The Feulgen technique used herein employed the same Schiff reagent as described by us for the Bauer method<sup>3</sup> and followed essentially the procedure outlined by de Tomasi<sup>4</sup> and by Stowell<sup>5</sup>: Deparaffinize and hydrate sections as usual. Rinse for one minute in N/1 HCl

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and hydrolyze for twenty minutes at 50° C. in N/1 HCl. Rinse for one minute in cold N/1 HCl and immerse for two hours in Schiff reagent. Give three successive ten-minute baths in closed Coplin jars in acid sulfite mixture (10 c.c. of 10 per cent NaHSO<sub>3</sub>, 10 c.c. of N/1 HCl, and 200 c.c. of distilled water). Wash for five minutes in running water. Counterstain briefly (thirty seconds) in 0.02 per cent alcoholic-fast green FCF, dehydrate in alcohols, clear in xylene, and mount in clarite.

The Gram-acetone-safranin technique was as published in 1928.<sup>6</sup> The Gram-Weigert technique is as follows: Deparaffinize and hydrate as usual. Stain for ten minutes in carbolfuchsin and wash in water. Stain for two minutes in ammonium oxalate crystal violet. Drain, blot, and apply three changes of Weigert's iodine to a time of thirty seconds total. Wash in water. Blot and decolorize with two or three changes of Weigert's aniline xylene (50:50), blotting between changes. Wash in three changes of xylene and mount in clarite.

#### OBSERVATIONS

*Protozoa.*—Muscle was resectioned for study from seven monkeys (*Macaca rhesus*), four more cats (*Felis cattus*), two Texas armadillos, one cotton rat (*Sigmodon hispidus*), one mouse (*Macaca musculus*), one ground squirrel (*Citellus beecheyi*), and seven wood rats (*Neotoma micropus*). By the Feulgen method the Sarcosporidia presented small oval red purple nuclei. They were Gram negative and Weigert negative. With the Bauer method there was much finely granular purplish red material about the nuclei of the parasites, so that the affected muscle fibers stood out conspicuously at very low magnifications.

Digestion with saliva, ptyalin, and malt diastase was resisted so that the granules remained Bauer positive in the first cat. Contrariwise, ptyalin and malt diastase digestions completely destroyed the Bauer-positive material in the armadillos, monkeys, wood rats, mouse, cotton rat, and two of the cats, thus identifying it as one of the hexose polysaccharides digestible by those enzymes. In the ground squirrel and the other two cats, most of the colonies lost their Bauer-positive material but some colonies and some individual organisms did not. That this increased resistance to digestion may have been artefact is indicated by the fact that Bauer-positive material remained after diastase digestion only in one of four sections from the ground squirrel tissues.

Further sections from the first cat were subjected to diastase digestion for periods of 30, 60, 90, and 120 minutes. While many Bauer-positive granules remained after 120 minutes, there was a very evident reduction in their number and size, and some cysts contained some or all Bauer-negative parasites. Shorter digestion periods left more and more Bauer-positive material. Altogether it appears that the positive Bauer reaction of these granules in the Sarcosporidia depends on glycogen or a similar polysaccharide.

The brains of nine mice, infected experimentally with toxoplasmosis and fixed in Orth's fluid, were resectioned. In four no parasites were found. In five microcysts containing *Toxoplasma* were identified. The Feulgen reaction demonstrated numerous tiny oval vesicular red purple nuclei in all five. In three of these the Bauer reaction showed many rather vague purplish red granules in a pale blue matrix. In the other two the parasites were apparently Bauer negative. Gram-acetone and Gram-Weigert stains were done on the three Bauer-positive specimens. Parasites were red and pale pink, respectively, by these methods.

Goodpasture stains were done according to Perrin's technique<sup>7</sup> and in the three brains in which parasites could be found they were stained brown.

After digestion tests with ptyalin and with diastase, *Toxoplasma* still stained red with the Bauer method. The Bauer-positive material was perhaps more clearly demonstrated as a deep purplish red perinuclear deposit, sometimes masking the nucleus; than in undigested material.

Encephalitozoa were found in six of seven mouse brains resected for study. They occurred as oval plump organisms in focal lesions. Some in the Gram stain are longer and slenderer. With this Gram-acetone method they stained pale to fairly deep violet but not blue black. With the Gram-Weigert method the organisms varied from red through purple to violet and sometimes blue black. Feulgen preparations from adjacent sections failed to demonstrate the organisms in the same focal lesions. In Bauer preparations the cells of the focal glioses assumed a vague pink color. Among them there were discerned with difficulty clear, oval refractile bodies of the same size and position as the organisms in Gram-Weigert and Goodpasture preparations. After diastase or ptyalin digestion, however, the organisms were more clearly discerned as oval purplish pink rings with clear centers lying in a light blue background.

The material for study of Encephalitozoon and *Toxoplasma* was resected from animals inoculated with the respective organisms by Perrin.<sup>7</sup>

Examples of endoerythrocytic and exoerythrocytic forms of *Plasmodium gallinaceum* were furnished me by Dr. L. R. Hershberger. In agreement with his usual findings the parasite nuclei stained purplish red by the Feulgen technique. Gram, Weigert, and Bauer reactions were negative.

Sections were recut from the kidneys of twelve mice infested with *Klossiella muris*. In eleven of these more or less numerous parasites were demonstrated. Rosette forms with peripheral budding showed in the buds small oval leptochromatic vesicular nuclei staining light red purple with Feulgen. Clusters of small intratubular round forms were Feulgen negative or contained faintly stained very fine single to multiple granules. In somewhat larger forms Feulgen-positive granules were still lightly stained and usually multiple. In medium-sized round forms dense red purple diplococcoid granules were present dispersed through the cytoplasm. Organisms of this last size were sometimes red purple or less often blue black with the Gram-Weigert technique. Sometimes as many as one-fourth to one-third of the individuals in a given intratubular cluster were so stained, while the rest were Gram negative. With the Gram-acetone method fewer organisms were stained violet or, occasionally, blue black, while other organisms in the same intratubular cluster presented multiple diplococcoid chromatin granules stained red by the safranin counterstain. By the Bauer method most organisms in all animals were Bauer negative. However, in all but one mouse, irregular masses and medium to fine granules of brilliant purplish red material were found in occasional to moderately numerous medium-sized parasites. Liver sections on the same slide in six of eleven mice showed moderate amounts of Bauer-positive glycogen in the liver cells.

Kidney sections from nine of these mice were successfully carried through ptyalin and diastase digestion tests, and the subsequently performed Bauer

reaction revealed in four of them occasional to moderately numerous parasites containing brilliantly purplish red granules. The six livers previously noted had lost all of their glycogen after the diastase digestion and all, or all but traces, after ptyalin.

*Trypanosoma equiperdum* in methanol-fixed thin blood films is Bauer negative, Weigert negative, and Gram negative. Trophonuclei stain pale purplish red with Feulgen and blepharoplasts were not discerned. However, lymphocyte nuclei are also rather lightly stained. Other fixations might give better results.

*Trypanosoma cruzi* was demonstrated only in one monkey heart and in two mouse hearts of several resectioned for study. The organisms occurred as the usual *Leishmania* forms in cysts within muscle fibers. Their cytoplasm stained light green, trophonuclei light red, and blepharoplasts as deep red purple rods in Feulgen-fast green FCF preparations. In Gram-acetone-safranin stains blepharoplasts were deep red, trophonuclei light red, and cytoplasm pink. With the Gram-Weigert technique cytoplasm and trophonuclei were pale pink and blepharoplasts deep violet to blue black. The Bauer reaction was negative, though a moderate amount of glycogen was shown in a liver section on the same slide with the one mouse heart and in muscle fibers of the other mouse heart.

Eight cases of amebiasis were resectioned for study. *Endamoeba histolytica* was readily found in all. The organisms were Gram negative and Weigert negative. The nuclei stained relatively faintly by the Feulgen technique. Many amebae contained more or less copious Bauer-positive granular material in their cytoplasm, but in some organisms the cytoplasm was blue gray and vacuolated and in some contained only a few purplish red granules. After diastase digestion all presented blue gray cytoplasm.

This observation extends that of Meriwether<sup>8</sup> who reported the selective staining of amebae by Best's carmine but did not identify the Best-positive material as glycogen. It would appear from the present observations that *Endamoeba histolytica* often contains glycogen or a similar polysaccharide.

Coccidia (*Eimeria stiedae*) were demonstrated in greater or less profusion in eight of eleven rabbit livers resectioned for study. With the Feulgen method most of the large coarsely granular parasites, whether intraepithelial or free, were Feulgen negative. Some small forms showed red purple granules or green granules with red purple margins. These were not found in five rabbits. In some rabbits there were found, in addition to the typical coccidia, masses of pale green cytoplasm without granules, in which were rings or spirals of small red purple fusiform or bacilliform chromatin granules. With the Gram-acetone technique granules were stained red and shells pink. The Gram-Weigert method gave blue black or deep violet coarse granules in the coccidia, while their shells were stained in varying tones from pink through violet to deep violet or blue black. The Bauer reaction demonstrated a pink to purplish red matrix in the coccidia, in which the refractile or unstained coarse granules were discernible. This material was present also in the coccidia in livers collected six to forty hours post mortem, in which all glycogen had disappeared from the liver cells. Application of Bensley's salivary digestion test to collodionized sections removed the pink matrix in two rabbits, at the same time removing all of the fairly copious



glycogen from the liver cells. In a third rabbit the same test apparently decreased the intensity of the Bauer reaction in the coccidial cytoplasm. Use of the diastase and ptyalin digestion tests on uncollodionized sections also removed Bauer-reacting material from nearly all of the parasites. All glycogen in liver cells was removed by the enzymes.

*Bacteria*.—Various sections of acute ulcerative appendicitis, of chronic appendicitis, and of animal stomachs and intestines, with or without acute ulcerative lesions, were examined. Various Gram-positive and Gram-negative bacilli and cocci were demonstrated with Gram and Weigert techniques. Gram-positive organisms were more plentiful with the Weigert method. These organisms all failed to stain by the Feulgen and Bauer techniques. However, a few Bauer-positive, Gram-negative branching filamentous molds were seen in a slough in amebic dysentery and in the margin of a cutaneous blastomycotic sinus. Material from five cases of human anthrax was included. The bacilli were usually partly Gram positive and partly negative with the Gram technique. The organisms were Bauer negative. A *Bacillus subtilis* contamination of lung tissue revealed numerous Gram-positive bacilli by the Gram and Weigert methods. The organisms were Feulgen negative and Bauer negative.

A series of four mice inoculated intracerebrally with material under study for the presence of poliomyelitis virus developed empyema of the cerebral ventricles. The pus cells contained a variable small amount of glycogen which was destroyed by the diastase digestion test. In the pus were clumps of small bacilli stained light blue with deep purple (metachromatic) polar granules by the azure eosin stain. With the Weigert stain the bacilli were barred, beaded, bipolar, or solidly blue black. The Bauer method revealed them as slender purplish red rods, which were possibly more distinct after diastase digestion than without it. The Feulgen method failed to stain these diphtheroid bacilli.

Guinea pig spleens and lungs containing numerous tubercle bacilli by the Ziehl-Neelson technique showed no organisms with Feulgen, Bauer, or azure-eosin methods. The bacilli stain blue black by the Weigert and Gram techniques if exposed a sufficient length of time or if heated in the crystal violet solution.

Four chick embryo yolk sacs fixed in Regaud's fluid were resectioned. Azure-eosin stains demonstrated numerous *Rickettsia orientalis* in all. These were not stained in otherwise good Feulgen or Bauer preparations. Tissues of four psittacine birds dead of acute psittacosis were resectioned. Numerous *Rickettsia psittaci* (LCL bodies) were demonstrated with azure eosin; none were demonstrated with the Feulgen method. Fresh sections from four rodents dead of acute tularemia presented numerous organisms in azure-eosin preparations; none presented organisms in Feulgen stains.

*Fungi*.—Histoplasmosis (*Histoplasma capsulatum*) was studied in the liver and spleen of two dogs and two hamsters, in the lung of a guinea pig, and in the omentum and abdominal wall of a rat. Fixation was with 10 per cent formalin, Helly, or Zenker, all three in one dog. The Feulgen method revealed tiny vesicular light red purple nuclei separated by a clear zone from a pale green capsule. In some of the Zenker-fixed material these nuclei were not demonstrated by Feulgen and stained poorly in comparison with those in formalin-

fixed material from the same dog with the iron-hematoxylin-van Gieson technique. With the Gram-acetone-safranin technique histoplasma nuclei usually stain red to pink; capsules stain paler or much paler. In the rat and in the liver of one hamster a few organisms stained blue black or violet, but most of them were Gram negative. A variable proportion of the organisms were Gram positive when the Gram-Weigert technique was used: none in one animal, few in another, one-third in one, "many" in two, and one-half to two-thirds in one. Usually Gram-positive organisms were stained solidly blue black; however, sometimes only the capsules were stained blue black or violet, while nuclei or nuclei and cytoplasm were stained red by the fuchsin pre-stain.

Results with the Bauer reaction were striking. Organisms were readily identified as fine red purplish rings surrounding a clear zone about the centrally placed, blue or pale blue nucleus. Organisms were more readily found than with azure-eosin or Giemsa stains. After salivary digestion the histoplasma capsules were still Bauer positive. Ptyalin and diastase digestion tests gave the same results in the five animals so tested.

In another subsequently studied animal numerous Bauer-positive rings without central nuclei were found in the foam-cell granulation tissue where no histoplasma could be found with azure-eosin stains. In other areas where histoplasma was demonstrable with the usual azure-eosin stain, the Bauer-positive rings were present in equal numbers and here contained the central blue stained (hematoxylin) nucleus as previously described. This indicates that the sulfurous acid-fuchsin method may be used to demonstrate non-nucleated and presumably dead histoplasma as well as the nucleated and presumably surviving forms. I am indebted to Dr. L. L. Ashburn for the observations on this dog and for material.

Five animals infected with *Haplosporangium parvum* were studied. The tissue studied was lung and was fixed in 10 per cent formalin in four of the animals and in Orth's fluid in the fifth. The animals comprised one rhesus monkey, one cotton rat, and one white mouse, all infected in the laboratory, and two *Perognathus* mice captured in the infected state. In the first three all of the organisms were Gram negative; in the last two a few were Gram positive, most Gram negative with the Gram-acetone technique. With the Gram-Weigert technique a few blue black organisms appeared in one of the first three animals, while in the last two most of the fungi were blue black. The Feulgen reaction revealed no Feulgen-positive material in any of the fungi, though host nuclei were well stained. With the Bauer reaction the capsules of the fungi appeared as thick deep purplish red rings, both with and without antecedent salivary digestion.

Subcutaneous masses from three guinea pigs, fixed in formalin, and brains from 3 mice, fixed in Orth's fluid, were studied in torulosis. In all numerous *Torula histolytica* were present. From 10 to 50 per cent of the yeasts were Gram positive with the Gram-acetone method. The Gram-Weigert method showed nearly all blue black in the three guinea pigs. It was not used on the mouse brains. In the guinea pigs the fungi were apparently Feulgen negative, while in the mouse material small round or oval nuclei were found. These

stained light red purple to pale lilac. With the Bauer method the outer shells of the yeasts appeared as deep purplish red to purplish violet rings. These were apparently somewhat redder in sections subjected to preliminary salivary digestion. Small forms appeared as solid red globules.

A *Peromyscus* mouse and seven white mice experimentally infected with *Coccidioides immitis* were reused for this study. Thirteen blocks from omental and visceral masses, testes, and lungs were used. Fixation was with buffered 10 per cent formalin for the seven white mice and with Orth's fluid for the *Peromyscus*. The fungi were regularly Feulgen negative except on one trial where pink nuclear staining was obtained, and a deteriorated Schiff reagent may have been used for this. Fungi were red by the Gram-acetone-safranin method except for a few blue black endospores in one and a few violet fungi in two of the thirteen blocks. With the Gram-Weigert technique a variable proportion were blue black or deep violet, from as few as one-fourth to nearly all in the several blocks studied. Endospores and small fungi were more often Gram positive than large forms or empty shells. Regularly the thin shells of the mature fungi and the shells of endospores and small fungi were red to red purple with the Bauer technique, and often the fungi contained solid purplish red masses within them, both endospores and larger forms. The five livers observed were glycogen free.

Application of ptyalin and diastase digestion tests to material from five animals reveals that the organisms still presented purplish red shells and endospores when the Bauer reaction was applied after the digestion, but no internal masses staining red persisted.

In Case A-2333, *Monilia candida* endocarditis, numerous small oval yeasts were demonstrated in the mitral thrombus. The yeast cells stained green with the Feulgen method. Within the cells were indistinct or distinct fine granules or delicate tiny rings of material staining purplish pink to red. The yeast cells were nearly all Gram positive by the Gram technique; all stained blue black by the Weigert method and deep purplish red by the Bauer method. After diastase digestion the positive Bauer reaction was unaltered.

Three cases (S-454, S-1187, and S-9410) of cutaneous blastomycosis and one case (A-888) of blastomycotic pulmonary abscesses were studied. The yeast cells were either Feulgen negative, or questionably pale, or very pale red purple nuclei were present. The yeasts were Gram negative, and, by the Weigert technique, only occasional cells stained violet in one of the four cases. The cell walls were stained deep purplish red by the Bauer method, both with and without preceding diastase digestion. Glycogen in epidermal cells and sweat gland cells was destroyed by the diastase.

Kernohan<sup>9</sup> has previously indicated the presence of a substance stained by Best's carmine method in the capsules of *Histoplasma capsulatum*, *Coccidioides immitis*, *Torula histolytica*, *Blastomyces hominis*, *Mucor*, and *Monilia*. The present study parallels but does not repeat these findings and further identifies the capsular substance as one resisting enzyme digestion with diastase and ptyalin.

A section of guinea pig omentum with experimental mycetoma was studied. The fixation was with 10 per cent formalin. The organism was *Allescheria boydii*. The tissues contained compact rings of closely felted, refractile brownish yellow, tortuous septate mycelia. The enveloping membrane was more often brown, sometimes blue black, with the Gram-Weigert method. It was unstained by the Gram-acetone method. The Bauer method showed most of the filaments in their natural brown. A few of the most distal, ramifying filaments were red, with or without antecedent diastase digestion. No chromatin was demonstrable with the Feulgen method.

Formalin-fixed liver and spleen from a rabbit infected with *Aspergillus fumigatus* presented Gram-negative mycelia with the Gram-acetone technique. With the Gram-Weigert method part of the mycelia were blue black and part red. With the Bauer method a very delicate red to purple capsule was recognized outside a violet protoplasm and crossing at the septa. This Bauer-positive material resisted salivary and diastase digestions.

The mycelia of chromoblastomycosis in the formalin-fixed omentum of a rat were partly blue black, partly red, and partly brown by the Gram-Weigert method. Staining was apparently restricted to an outer capsular substance, so that the mycelia appeared as hollow tubes. With the Gram-acetone and the Bauer techniques the mycelia apparently retained their natural brown color for the most part. Some filaments and, especially, spore-like enlargements presented Bauer-positive walls, particularly after diastase digestion.

In Case S-8442, mycetoma of wound of hand, the granules were Feulgen negative and Gram negative with the Gram-acetone method. By the Gram-Weigert technique there were few to moderately numerous ramifying septate Gram-positive mycelia. These appeared foamy, vacuolated, or barred blue black on a pink background. With the Bauer method the granules were composed of a dense felt work of light purplish red hollow, rather thin-walled tubules, which remained Bauer positive after diastase and ptyalin digestion.

In Case S-14788, *Cephalosporium* mycetoma, the granules were again Feulgen negative and Gram negative with the Gram-acetone method and again contained a few branching, foamy, vacuolated, or solid blue black mycelia by the Gram-Weigert method. The Bauer reaction again revealed a close feltwork of light red purple tubules, which remained Bauer positive after diastase digestion.

In Case A-2884, *Cephalosporium* mycetoma, multiple sections of sinus tracts presented numbers of large granules composed of wide dichotomously and laterally branching mycelia with frequent transverse septa and scattered bulbous enlargements to twice ordinary width and about thrice the mycelial width in length. These bulbous enlargements were relatively thick walled. The margin was partly composed of bulbous enlargements similar to the foregoing in structure. The mycelia and bulbs were Feulgen negative and Gram negative. With the Weigert stain part of the mycelia were stained pink, part presented violet walls, and some presented transverse blue black bars. Some violet walled mycelia ramified beyond the hyaline Gram negative-matrix into the surrounding

pus. With the Bauer method the mycelial walls and septa stained deep purplish red, with or without antecedent diastase digestion. The mycelia were not acid fast.

In Case A-3072, *Penicillium* species abscess of lung, imbedded in the pus of the abscess cavities and in giant cells were fragments and radiating stellate masses of broad septate mycelium with closely spaced septa, scattered bulging thicker-walled segments, and separate oval yeast-like globules similar in short diameter to the bulging segments. Lateral and dichotomous branching were observed. Mycelia were "negative" to the Ziehl Neelson, Feulgen, and Gram methods. Most filaments stained pink; a few stained violet, and occasional ones were solidly or barred blue black by the Weigert method. Bauer stains, both before and after diastase digestion, colored the walls and septa a deep purplish red.

Four cases of actinomycosis (A-478, A-1914, S-12263, and A-2187) and one case of maduramycosis (S-17813) presented more or less numerous granules composed of a central feltwork of narrow mycelial threads and a peripheral hyaline matrix whose outline varied from granule to granule in each case from smooth or lobate to tongue-like narrow prolongations with bulbous tips. The mycelial threads only infrequently presented dichotomous branching. They were Feulgen negative. Many stained blue black, some solidly, some barred or beaded, and some red with the Gram method, and nearly all were blue black by the Weigert method. The hyaline matrix and border was Gram negative by both methods. With the Bauer method some granules presented a vague purplish pink granular deposit centrally; in some this purplish pink material was vaguely filamentous, and in a few more sharply defined purplish red filaments were discerned. After diastase digestion vague and definite filaments were often more readily discerned.

Of the foregoing cases, only the last was studied culturally.

In Case S-22853, mycotic gastric ulcer, the Gram-acetone method revealed a profusion of red filaments and fine and coarse rods in the ulcer base. A minority of these organisms were blue black by the Gram-Weigert method. The filaments and rods were unstained by the Feulgen and Bauer methods.

Sufficient material from one case only of botryomycosis (S-11861) was available for restudy. One to several "granules" were found in suppurating sinuses in each of the five sections. Azure-cosin stains revealed a hyaline eosinophilic border zone, marginally serrate, lobate, or bulbous. Next within this was a zone of densely packed cocci, sometimes poorly stained and sometimes deep blue. The centers were composed of more or less closely packed, deeply basophilic cocci lying in clusters, pairs, or short chains. With the Gram stain the cocci were Gram positive in some granules and Gram negative in others. By the Weigert method there were always considerable numbers of closely and loosely packed Gram-positive cocci occurring in clusters and lying in a hyaline pink matrix. The marginal serrate or club zone was hyaline and stained pink to fairly deep red.

By the Bauer method, with and without antecedent diastase digestion, the granules stood out under low magnification as conspicuous purplish red

masses. The border or club zone stained pale gray or blue gray. The cocci appeared as pale blue bodies. Among the cocci were numerous fine red granules and short and narrow red bacilli, fewer pale pink rods containing red granules, and a few longer red filaments which occasionally showed branching. Bacillary and filamentous structures were best discerned in marginal areas. Central areas presented too much vague red granular material to permit discernment of detail. Occasionally coarser pink filaments with clear centers were discerned, and in cross section these appeared as pink to red rings about the same size as the cocci. Red filaments and bacilli were perhaps  $0.5\ \mu$  in thickness; cocci were about 1 to 1.5 microns.

The existence of small bacillary and occasionally filamentous Bauer-positive elements in the granules of this case of botryomycosis in addition to the usually described Gram-positive staphylococci is a matter of interest requiring further investigation to decide as to its possible etiologic significance.

*Metazoa*.—Tissues from eight animals were resectioned for study and Schistosome eggs were demonstrated in seven of them. Two species, *Schistosoma mansoni* and *Schistosoma japonicum*, were included in the material, and the animals included six experimentally infected hamsters and a naturally infected dog. Some adult parasites were found in veins. Nuclei of adult parasites and embryos were red purple by the Feulgen method, just as were the host cell nuclei. With the Gram-acetone technique the shells were usually pink but sometimes pale violet. With the Gram-Weigert method the shells varied from pink to blue black, being perhaps most often violet. Using the Bauer technique the shells were usually deep purplish red. Fragmenting shells stained similarly and the granulomata surrounding fragmenting eggs or replacing them often contained globules and masses of purplish red to purplish pink hyaline material in the cytoplasm of the epithelioid cells. After diastase and ptyalin digestion tests the shells of the ova still stained red purple with the Bauer method, but the cytoplasm of the epithelioid cells became Bauer negative.

The implication that the cells of the granuloma convert the chitin of the Schistosome eggshells into a polysaccharide which is digestible by ptyalin and by diastase, while the chitin itself is not so digestible, appears to warrant further study.

The livers of four wild rodents infected with numerous doubly operculate eggs of *Capillaria (Hepaticola) hepatica* were resectioned for study. The infected animals were a woodchuck (6240), a cottontail rabbit (8385), a jackrabbit (8788), and a squirrel (8789) from Western Montana. These ova, when stained with azure eosin, presented several blue nuclei, coarsely granular pink cytoplasm, a hyaline, refractile inner shell, and a refractile, radially striate outer shell. The operculae appeared as apparently open spaces. The embryo nuclei stained purplish red by the Feulgen method, the refractile inner shell was tinted yellow to orange, and the cytoplasm and the radially striate outer shell stained green. With the Gram stain the refractile inner shell stained pink to violet, the cytoplasm was largely pink, sometimes containing violet granules, and the outer shells were refractile to pale pink. The nuclei stained red. With the Weigert method the refractile inner shell stained deep red, the operculae were filled by

deep violet to blue black plugs, the cytoplasm was violet to pink, and the radially striate outer shell was pink. Diastase and ptyalin digestions did not appreciably alter these reactions.

Inner and outer shells remained unstained or yellowish by the Bauer method, the operculae were filled by deep purplish red plugs, nuclei were blue, and cytoplasm was granular and light purplish red or less often light blue. Diastase digestion rendered the cytoplasm of most of the eggs Bauer negative; ptyalin was less effective but still destroyed most of the Bauer-positive granular material in the cytoplasm. The opercular plugs still stained deep purplish red after digestion with either enzyme.

In a Norway rat (1364), cysticereosis of liver, most of the cyst walls and cestode tissues were Gram negative, Weigert negative, and Bauer negative. In one section, apparently scolex, much granular purplish red material was present in the cestode tissue with the Bauer method, and the Weigert preparation gave violet granules in the same location. This section possessed the most Feulgen-positive nuclei, but fair numbers of these were present also in the cyst walls. After ptyalin and diastase digestion tests the Weigert-positive material was destroyed, and the Bauer-positive material in the scolex persisted after ptyalin but not after diastase digestion.

In a snowshoe rabbit tapeworm (15720), the calcareous globules were deep blue by azure eosin, violet with Weigert, and unstained by the other methods. Nuclei were stained purplish red with the Feulgen technique. The cuticle was deep blue with azure eosin and red with Weigert and Gram techniques. The Bauer preparation showed numerous granules and globules of purplish red material in the stroma. After a ptyalin digestion test this material became Bauer negative. Similarly, Bauer<sup>1</sup> has shown the presence of glycogen in stroma cells and elsewhere in *Fasciola hepatica*, using iodine, Best and Bauer methods, and salivary digestion tests with the two latter stains.

In Case S-4147, appendix containing *Trichuris* and *Ascaris* eggs, the eggs appear yellow by the Bauer method, brown to yellow by the Feulgen, pink to red by Gram-acetone, and their shells stained red to purple by Gram Weigert.

In Case S-18128, *Onchocerca volvulus* in skin, the chitin of adults stained pale reddish purple with the Bauer method, whereas the same parasites contained numerous Feulgen-positive nuclei. Feulgen-negative (dead?) parasites gave a denser red purple chitin stain with the Bauer method. The chitin was Feulgen negative, Gram negative, and Gram-Weigert negative.

#### SUMMARY

Sharply stained nuclei were demonstrated by the Feulgen method in the following: *Sarcosporidia*, *Toxoplasma*, *Klossiella muris*, *Trypanosoma cruzi*, *Plasmodium gallinaceum*; in eggs, larvae, and adults of *Schistosoma japonicum* and *Schistosoma mansoni*; in larvae and adults of tapeworms, roundworms, and *Onchocerca volvulus*; and in eggs and larvae of *Capillaria hepatica*. Poorly stained or dubiously Feulgen-positive nuclei were encountered in *Encephalitozoon*, *Eimeria stiedae*, *Endamoeba histolytica*, *Torula histolytica*,

*Blastomyces*, and *Histoplasma capsulatum*. The mycelial fungi, bacteria, rickettsiae, *Coccidioides immitis*, and *Haplosporangium parvum* failed to present Feulgen staining material.

Differences between the Gram-acetone method and the Weigert-aniline xylene-Gram variant were generally in the direction that the latter yielded more Gram-positive organisms. *Eimeria stiedae*, almost completely Gram negative by the acetone technique, regularly contained numerous conspicuous Weigert-positive granules. One-fourth to two-thirds of the cells of *Histoplasma capsulatum* in any given preparation were Gram positive by the Weigert method, while all were negative by the acetone method. *Haplosporangium*, *Torula*, and *Coccidioides* all showed many more Gram-positive cells with the Weigert method. *Klossiella muris* often presents Gram-positive cells by the Weigert method, seldom with the acetone method. *Encephalitozoon* is weakly positive by the acetone method, while negative and strongly positive cells are seen side by side in Weigert preparations. Mycetomal and other thick mycelial fungi often present some positive filaments with the Weigert method and none with the acetone. Weigert-positive, Gram-negative material is seen in the shells of *Schistosoma* eggs and in the opercular plugs of *Capillaria* eggs.

Bauer-positive polysaccharides digestible with ptyalin and malt diastase are present in the cytoplasm of *Endamoeba histolytica*, *Eimeria stiedae*, most Sarcosporidia, and in the egg cytoplasm of *Capillaria hepatica*.

Bauer-positive material which resists digestion by ptyalin and malt diastase forms the capsules of the following: certain yeasts, such as *Monilia candida*, *Histoplasma capsulatum*, *Torula histolytica*, *Haplosporangium parvum*, *Coccidioides immitis*, and *Blastomyces* species; certain coarsely mycelial fungi, such as those of mycetoma, *Penicillium*, *Aspergillus*, and others; the shells of the eggs of *Schistosoma mansoni* and *Schistosoma japonicum*; and the opercular plugs of the eggs of *Capillaria hepatica*. Bauer-positive, relatively enzyme-resistant granules are found in some Sarcosporidia, in a few individuals of *Klossiella muris*, and in *Toxoplasma*.

The ray fungi possess small quantities of diffuse material reacting to the Bauer test, which often appears more definitely filamentous after enzyme digestion tests. Similar bacillary and occasionally filamentous Bauer-positive material was found among the staphylococci in the granules in a case of botryomycosis. Some Gram-positive diphtheroid bacilli are also Bauer positive, with or without diastase digestion.

The Bauer method offers a valuable aid in detection of scarce amebae and yeastlike fungi in tissues. It gives structural details in the more coarsely mycelial fungi which are not apparent with other usual staining methods. It clearly demonstrates the remaining fragments of *Schistosoma* eggs in granulomata in which they are not readily identifiable by other means.

In its possession of a Bauer-positive, enzyme-resistant capsule and in its partially Gram-positive reaction *Encephalitozoon* shows a certain resemblance to *Coccidioides* and to *Histoplasma*. Wenyon<sup>10</sup> has stated that "in many respects it resembles a small yeast, but reproduction by budding has not been observed." He further states that "It seems premature to conclude that the organism is



even a protozoon." The present data tend to accentuate the doubt of the protozoal nature of this organism. It is noteworthy that both *Coccidioides* and *Histoplasma* were first thought to be protozoal.

#### REFERENCES

1. Bauer, H.: Mikroskopisch-chemischer Nachweis von Glykogen und einigen anderen Polysacchariden, *Ztschr. f. mikr.-anat. Forsch.* 33: 143-160, 1933.
2. Bensley, C. M.: Comparison of Methods for Demonstrating Glycogen Microscopically, *Stain Technol.* 14: 47-52, 1939.
3. Lillie, R. D., and Greco, J.: Malt Diastase and Ptyalin in Place of Saliva in the Identification of Glycogen, *Stain Technol.* In press.
4. de Tomasi, J. A.: Improving the Technic of the Feulgen Stain, *Stain Technol.* 11: 137-144, 1936.
5. Stowell, R. E.: Feulgen Reaction for Thymonucleic Acid, *Stain Technol.* 20: 45-53, 1945.
6. Lillie, R. D.: The Gram Stain. I. A Quick Method for Staining Gram-Positive Organisms in the Tissues, *Arch. Path.* 5: 828-834, 1928.
7. Perrin, T. L.: *Toxoplasma* and *Encephalitozoon* in Spontaneous and in Experimental Infections of Animals, *Arch. Path.* 36: 568-578, 1943.
8. Meriwether, L. S.: Identification of Amoebae in Tissue, *J. Tech. Methods* 14: 64-65, 1935.
9. Kernohan, J. W.: A New Staining Method to Demonstrate Pathogenic Yeasts and Fungi, *J. Neuropath. & Exper. Neurol.* 2: 95-98, 1943.
10. Wenyon, C. M.: *Protozoology*, New York, 1926, William Wood & Company, pp. 754-756.

# A SIMPLE METHOD OF STAINING MALARIA PARASITES

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THE report by Manwell\* incites me to report another simple method for staining malarial parasites which was used during the war when no Leishman, Wright, or Giemsa's stains were available and when buffered or distilled water could not be obtained.

In a 50 c.c. porcelain evaporating dish, place 0.5 Gm. methylene blue and 1.5 Gm. borax. Add, with stirring, 30 c.c. of boiling tap water. Place the evaporating dish over a small flame at such a height as to maintain a temperature of 75 to 80° Centigrade. The liquid evaporates almost to dryness in approximately one-half hour. Add 30 c.c. of boiling tap water for a second and a third time, evaporating after each addition. Set aside for twenty-four hours. A dry blue compact mass remains in the evaporating dish. Add, without stirring, 30 c.c. of 95 per cent alcohol. After five minutes pour the alcohol into a 100 c.c. bottle and let the residue in the evaporating dish dry almost completely. Repeat the extraction in the same way, once with 30 c.c. and once with 40 c.c. of alcohol. The total alcoholic extract (about 100 c.c.) is blue and contains no precipitate.

On the unfixed blood film, place 15 drops of the alcoholic stain. After thirty to sixty seconds add 15 drops of a 1 to 2000 solution of eosin in tap water. Mix the stains on the slide by means of a glass rod. After three to four minutes flood the slide with tap water, wash with tap water, and let dry. The red cells appear light green with a pinkish cast, the eosinophile granules stain pink, and the remaining leucocytes and the platelets stain as with Giemsa's stain. The malarial parasites also have the same appearance as when treated with Giemsa's stain but are stained somewhat less deeply.

The satisfactory results obtained by this method are probably due to: (1) nearly complete transformation of methylene blue to azure; (2) the greater solubility in alcohol of the azure as compared to the methylene blue solubility; and (3) the insolubility of borax in alcohol. The technique constitutes a combination and modification of older ones published by many authors.

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From the Pasteur Institute.

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\*Manwell, R.: The J. S. B. Stain for Blood Parasites, J. LAB. & CLIN. MED. 30: 1078, 1945.

# THE USE OF RUSSELL VIPER VENOM AS A MEANS OF FOLLOWING THE ANTICOAGULANT ACTION OF 3,3'-METHYLENEBIS (4-HYDROXYCOUMARIN) IN THE DOG

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WITH THE TECHNICAL ASSISTANCE OF ROCCO FANELLI

FULLERTON,<sup>1</sup> Page and Russell,<sup>2</sup> Shapiro and co-workers,<sup>3</sup> and others have shown that when Russell viper venom was substituted for thromboplastin in Quick's<sup>4</sup> prothrombin time test, certain points of resemblance could be noted. The purpose of this paper is to present comparative data obtained by the action of each of the two clotting accelerators on the plasma of dogs treated with 3,3'-methylene bis (4-hydroxycoumarin) (Dicumarol).<sup>5</sup>

Link and associates<sup>5, 6</sup> have shown that dicumarol is probably responsible for the hemorrhagic action of spoiled sweet clover hay. It is believed generally that the drug acts by reducing the effective prothrombin level. In fact, Quick's prothrombin time test was used to guide the isolation work. Without attempting to review the literature in the clinical field, it may be stated also that thromboplastin has been frequently employed to follow the course of dicumarol therapy.<sup>7-9</sup> Russell viper venom also has been used for this purpose, and among its users may be mentioned the names of Shapiro and Sherwin<sup>8</sup> and Wright and Prandoni.<sup>10</sup>

## METHODS

Blood was obtained from dogs by venipuncture, decalcified by mixing with 2 mg. of dry potassium oxalate per cubic centimeter of blood, and centrifuged promptly. The clear plasma was diluted by adding 3 volumes of 0.9 per cent NaCl and the prothrombin time determined on one portion with 2.5 per cent thromboplastin as described by Quick.<sup>4</sup> A commercial preparation prepared from rabbit brain was used for this purpose. This concentration of plasma (25 per cent) was found to be well suited for use with Russell viper venom since it yielded times of convenient length. Much longer times were obtained with thromboplastin at the maximum dicumarol effect. It may be noted that times may be shortened for both thromboplastin and Russell viper venom by increasing the plasma concentration or they may be lengthened by diluting the plasma.

A second portion was tested with Russell viper venom instead of thromboplastin. In carrying out this determination, 0.1 c.c. of the 25 per cent plasma was mixed with 0.1 c.c. of 1:10,000 Russell viper venom† and 0.1 c.c. of 1 per

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\*Dicumarol is the trademark for a commercial preparation of 3,3'-methylene bis (4-hydroxycoumarin).

†Supplied as Stypven Brand Russell Viper Venom by Burroughs Wellcome & Co. (U. S. A.) Inc., New York, N. Y.

cent calcium chloride was then added. A stop watch was started at the instant of the addition of the calcium chloride and stopped when the first flecks of fibrin appeared. For the sake of convenience this elapsed time interval will be known as the RV time. All determinations were made in triplicate. Eight different dogs were used in these experiments.

Dicumarol in gelatin capsules was given orally.

### RESULTS

The results of a typical experiment are given in Table I. It will be observed that the RV times paralleled the prothrombin times in several respects. Both coagulating agents simultaneously revealed the onset of dicumarol action, both indicated about the same duration of action, and both showed peaks of maximum intensity at about the same time. It will be observed that the thromboplastin figures rose to relatively higher values. Also thromboplastin figures fell to relatively lower levels during the reaction period which lasted for two or three weeks after the dicumarol administration.

TABLE I. RELATIONSHIPS BETWEEN DICUMAROL ADMINISTRATION, PROTHROMBIN TIME, AND RV TIME IN DOG

DATE	MEAN RV TIME (SECONDS)	MEAN PROTHROMBIN TIME (SECONDS)	DICUMAROL DOSAGE (MG. PER KG.)
11/15	28.3	31.1	10
11/16	40.5*	57.3*	10
11/17	46.6*	314.0*	10
11/18	71.4*	>900.0*	--
11/20	107.0*	>900.0*	--
11/22	62.4*	182.0*	--
11/24	40.3*	31.3	--
11/25	28.0	34.1	--
11/27	31.3	30.4	--
12/11	21.8	18.3	--
12/12	23.2	17.1	--
12/14	21.2	13.1	--
12/16	24.0	14.7	--
12/18	24.8	13.9	30
12/19	38.8*	26.9*	--
12/20	49.8*	56.3*	--
12/21	50.3*	121.0*	--
12/22	56.3*	433.0*	--
12/23	61.8*	133.0*	--
12/26	34.5*	20.2	--
12/27	29.2	17.8	--
12/29	26.6	17.7	--
1/1	29.8	20.1	--

All determinations were done in triplicate on 25 per cent dog plasma.

\*Values significantly above the normal level.

These experiments confirmed the earlier observations of Butt and associates<sup>7</sup> that divided doses of dicumarol were more effective than the same quantity administered in a single dose.

In considering the significance of the effects produced by dicumarol, account must be taken of the reproducibility of the test and the fluctuations in normal levels. Both thromboplastin and Russell viper venom gave highly consistent values. The total range experienced in triplicate determinations is rarely more than one or two seconds. Normal times, however, vary from

dog to dog, and Fig. 1 gives an example of how times may vary within the same animal. If the data of November 15, 25, 27, December 11 to 18, 27, 29, and January 1, as given in Table I, be considered as normal values, it will be seen that the RV times for this animal varied about a mean of 26.2 seconds. The standard deviation of these eleven values was 3.4 seconds. It is commonly assumed that any sample which breaks through a range embraced by the mean  $\pm 2$  times the standard deviation should be considered significantly different. In this case the upper limit would be  $26.2 + (2 \times 3.25)$  or 32.7 seconds. If this figure be taken as a guide, it is apparent that the administrations of dicumarol were followed by significant increases in RV times.

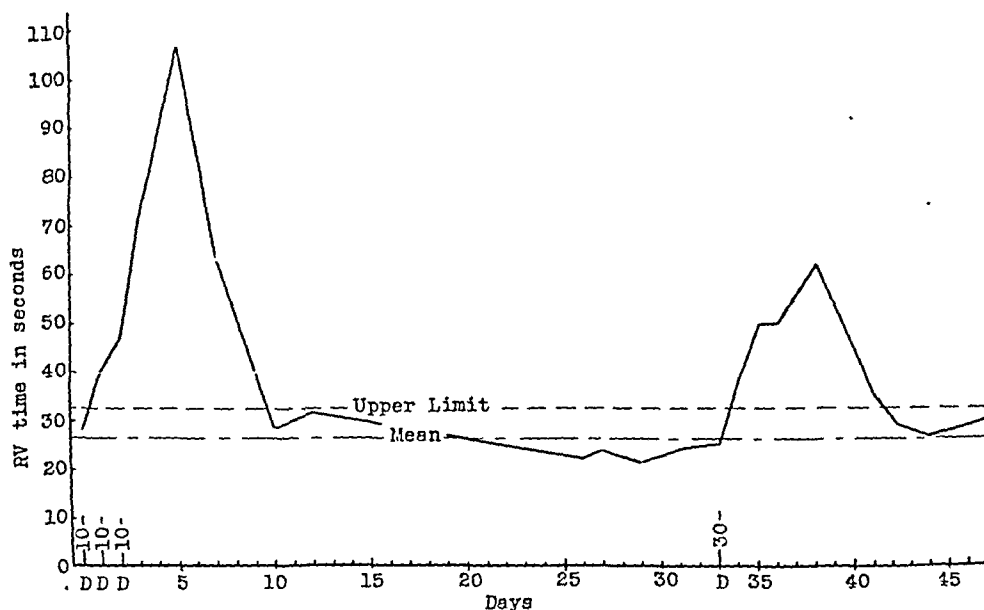


Fig. 1.—The effect of dicumarol on the RV time of dog plasma. D, Dicumarol in milligrams per kilogram.

#### SUMMARY

Russell viper venom resembled thromboplastin in its ability to follow the course of the anticoagulant action of 3,3'-methylene bis (4-hydroxycoumarin) in the dog.

The times for the formation of clots by thromboplastin were relatively longer than the times for the formation of fibrin flecks by Russell viper venom.

#### REFERENCES

1. Fullerton, H. W.: Estimation of Prothrombin; a Simplified Method, *Lancet* 2: 195, 1940.
2. Page, R. C., and Russell, H. K.: Prothrombin Estimation Using Russell Viper Venom. I. Simple Modification of Quick's Method, *J. LAB. & CLIN. MED.* 26: 1366, 1941.
3. Shapiro, S., Sherwin, B., Redish, M., and Campbell, H. A.: Prothrombin Estimation; a Procedure and Clinical Interpretations, *Proc. Soc. Exper. Biol. & Med.* 50: 85, 1942.
4. Quick, A. J.: *The Hemorrhagic Diseases and the Physiology of Hemostasis*, Springfield, Ill., 1942, Charles C Thomas, Publisher.

5. Campbell, H. A., and Link, K. P.: Studies on the Hemorrhagic Sweet Clover Disease. IV. The Isolation and Crystallization of the Hemorrhagic Agent, *J. Biol. Chem.* 138: 21, 1941.
6. Stahmann, M. A., Heubner, C. F., and Link, K. P.: Studies on the Hemorrhagic Sweet Clover Disease. V. Identification and Synthesis of the Hemorrhagic Agent, *J. Biol. Chem.* 138: 513, 1941.
7. Butt, H. R., Allen, E. V., and Bollman, J. L.: A Preparation From Spoiled Sweet Clover [3,3'-Methylene-bis-(4-hydroxycoumarin)] Which Prolongs Coagulation and Prothrombin Time of the Blood; Preliminary Report of Experimental and Clinical Studies, *Proc. Staff Meet., Mayo Clin.* 16: 388, 1941.
8. Shapiro, S., and Sherwin, B.: Studies in Thrombo-Embolization. II. Observations on the Use of Dicumarol (3,3' Methylenebis [4-hydroxycoumarin]) in Embolization. Report of 5 Cases, *New York State J. Med.* 43: 45, 1943.
9. Hurn, M., Barker, N. W., and Magath, T. B.: Prothrombin Time Determinations Following the Administration of Dicumarol With Special Reference to the Variability of Thromboplastin, *Proc. Staff Meet., Mayo Clin.* 19: 507, 1944.
10. Wright, I., and Prandoni, A.: The Dicoumarin 3,3'-Methylene-bis-(4-hydroxycoumarin); Its Pharmacologic and Therapeutic Action in Man, *J. A. M. A.* 120: 1015, 1942.

## MICROANALYSIS OF OPIATES BY X-RAY DIFFRACTION\*

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SINCE the development of various derivatives of morphine and the ultimate study of many of them in man and animals, it has become more difficult to find chemical tests suitable for their identification and determination in amounts present in biologic materials after administration. Occasionally toxicologic examinations of the lesser known opium derivatives require other than chemical means for a specific and conclusive identification.

The x-ray diffraction method has been used successfully for many years as a critical, characteristic means of identification, but it has not found application in many fields where only extremely small amounts of materials are available for analysis. Its advantages have led us to develop microdiffraction techniques of sufficient sensitivity that practically any sample of crystalline material may be identified and determined quantitatively. The new methods of identification serve not only to adapt small samples for diffraction information but include topochemical reactions in the preparation and examination of derivatives.

### METHODS

The flat cassette method of x-ray diffraction was employed in the present experiments (Fig. 1). The x-ray beam, collimated to a thin pencil of radiation about 0.010 inch in diameter, was allowed to penetrate the sample, and the diffraction pattern was recorded on a flat film mounted perpendicularly to the x-ray beam and separated from the sample by 5 centimeters. The sample holder was a small metal disk perforated with an opening  $\frac{1}{64}$  inch in diameter in which the sample (about 0.08 mg. or 80  $\gamma$ ) was packed. X-radiation from a copper target diffraction tube, filtered through 0.001 inch nickel foil, was used; exposures were two hours at 14 ma. and 40 kv. peak. Data are tabulated in terms of interplanar spacings or "d" values, where "d" is defined by the Bragg law,

$$d = \frac{\lambda}{2 \sin \Theta}.$$

The wave length of x-radiation used ( $\lambda$ ) is essentially 1.54 A.U.;  $2 \Theta$  represents the angular deviation of the diffracted x-ray beam from the undiffracted beam (Fig. 1). Intensity values were estimated with a Leeds and Northrup recording microphotometer. No attempt was made to correct for absorption of the x-ray

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\*The x-ray work was carried out in the Department of Analytical Chemistry at the University of Illinois. The drugs used in the test were prepared by Dr. Lyndon F. Small, formerly of the University of Virginia and now at the National Institute of Health, while participating in the work of the Committee on Drug Addiction of the National Research Council.

†Formerly with the Noyes Laboratory of Chemistry, University of Illinois; now with the General Aniline and Film Corporation, Easton, Pa.

‡Formerly Biological Chemist, Research Department, United States Public Health Service Hospital; now with the Wm. S. Merrell Company, Cincinnati, Ohio.

beam in the samples, although samples were made as thin as possible to obviate this factor. Intensity values listed are comparable only with patterns made by the flat cassette method, although "d" values are identical within experimental error with those obtained from any type of x-ray powder diffraction pattern technique.

A single pattern is acceptable identification when exact coincidence with a reference is obtained, but the use of two patterns of an alkaloid (one of the base and one of its hydrochloride salt) renders the result conclusive. Such reactions are readily carried out topochemically as follows: After a pattern of the free base is obtained, the mounted sample may be exposed to hydrochloric acid fumes for a short time. This converts the base to the hydrochloride salt (as shown by the absence of the diffraction lines of the base), and the second diffraction pattern may be obtained. If the starting material were the hydrochloride, exposure to ammonia fumes will convert it to the free base.

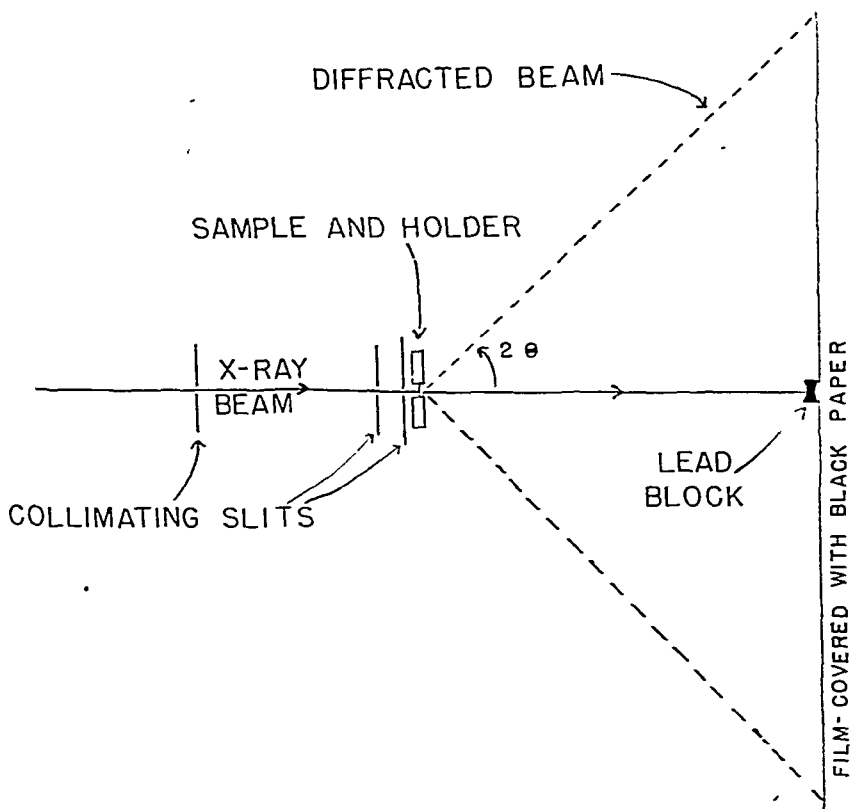


Fig. 1.

These gas treatments are conveniently carried out by rinsing a beaker with concentrated hydrochloric acid or with ammonium hydroxide, as desired, and inverting it over the sample mounted in the holder for twenty to thirty minutes. The sample holder must be constructed of material which will not react with the fumes; otherwise, it is necessary to remove the sample from the holder and



replace it later. The pattern of ammonium chloride formed in the sample will appear on subsequent diffraction patterns, but does not interfere with the identification.

### RESULTS

*X-ray Diffraction Patterns of Several Opiates and of Demerol.*—The data given in Table I are arranged for classification according to the system employed by Hanawalt<sup>1</sup> in his tables for the identification of unknown materials by the diffraction method. Roman numerals I, II, and III, following intensity values, correspond to the three strongest lines in the order of their intensity. Symbol B indicates that the line is unusually broad, and brackets are employed when two lines are so close together that slight changes in experimental conditions might make them appear as a single diffraction interference.

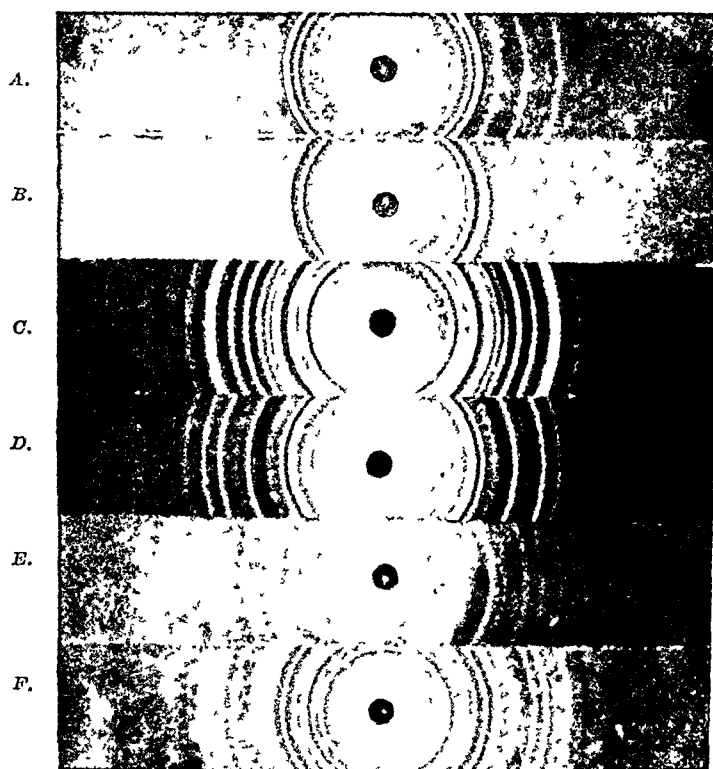


Fig. 2.—Patterns made with filtered copper K-alpha radiation; sample—film distance, 5 cm.; two-hour. exposure, 20 Ma. and 40 kv. A, morphine base; B, morphine hydrochloride; C, morphine sulfate dihydrate; D, morphine sulfate pentahydrate; E, heroin base; F, heroin hydrochloride.

The characteristic experimental measurements for a number of opiates and for Demerol\* are listed in Table I; example prints are shown in Fig. 2. Attention is directed to the patterns of two hydrates of morphine sulfate, the one be-

\*This drug was furnished by Dr. O. W. Barlow, formerly of the Winthrop Chemical Company, Inc., New York, N. Y.

ing a dihydrate and the other a pentahydrate.\* The pattern obtained after heating the pentahydrate of morphine sulfate between 185 and 195° C. for two hours is also included in the table. This pattern is reproducible and probably represents largely the anhydrous form, although it may also contain some other decomposition products. This pattern is not accounted for by combination of the two hydrates.

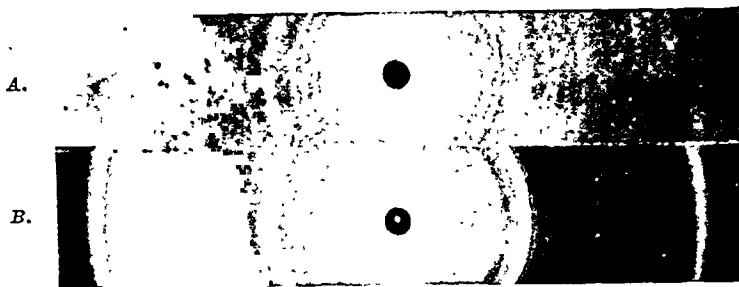


Fig. 3.—A, Diffraction pattern of urinary residue after heroin administration, showing presence of morphine (small amount of magnesium oxide added). Exposure made seven hours. B, Diffraction pattern of 0.7  $\gamma$  morphine suspended in 0.06 mg. magnesium oxide.

*Detection of Morphine in Urine by X-ray Method.*—In Fig. 3A is shown the diffraction pattern of an alkaloid present in a residue prepared from urine of an addict receiving heroin.† Comparison with Fig. 3B shows that this product is largely morphine; no heroin could be detected. This is in accord with excretion studies previously reported.<sup>3</sup> The total amount of sample available in this case was about 0.98 milligram. The spots on the pattern indicate that the material was present as crystals with particle size greater than about  $10^{-3}$  centimeters. Below this dimension the lines are sharp and smooth until colloidal dimensions are attained. In either case the position of the diffraction lines is unchanged.

Since urinary residues containing morphine, etc., are rather hygroscopic, they should be examined under conditions of reduced humidity.

#### DISCUSSION

The described method of identification by x-ray diffraction technique is accepted as definite, especially when based upon two diffraction patterns. It is not

\*The results on two hydrates of morphine sulfate are worthy of mention. Patterns obtained from a single commercial sample were not identical with those of Dr. Howard L. Andrews of the National Institute of Health. This stimulated further examination of seven additional samples; three of these gave the same pattern as ours and four were identical with those of Dr. Andrews.

Dr. Andrews<sup>2</sup> heated the pentahydrate of morphine sulfate at 57° C. for two hours and then made an x-ray pattern in a sealed tube. A mixture of the two patterns was obtained. Heating the pentahydrate at 60° C. for two hours gave a compound having a pure pattern agreeing with our original one. When the tube was left open for a few hours, the diffraction pattern returned to the form found for the pentahydrate. Weight loss determinations made by Dr. Andrews indicate that the change in patterns is accompanied by a loss of 3 molecules of water. It is believed that one pattern is obtained from the pentahydrate and the other from the dihydrate.

†The alkaloid excreted in urine was extracted by means of a chloroform-alcohol mixture (3 and 1 parts, respectively) according to the technique described by Oberst<sup>2</sup> using a liquid-liquid extractor. After evaporation of the solvent the residue was subjected to isoelectric extractions. The residue obtained after evaporation of the organic solvent was then treated with permutit to separate morphine from undesirable substances extracted. The permutit was washed and then treated with alkali to remove morphine which was re-extracted with the chloroform-alcohol mixture. After evaporation of the solvent the residue was weighed and submitted to x-ray examination.

TABLE I. INTERPLANAR SPACINGS AND RELATIVE INTENSITIES,\* USING A FLAT CASSETTE AND FILTERED COPPER K-ALPHA RADIATION

d A.U.	I	d A.U.	I
<i>1. Morphine Base</i>			
10.14	0.16	4.82	0.15
7.37	0.50 III	4.16	0.45
6.59	0.65 II	3.98	0.10
6.25	0.15	3.71	0.10
6.00	1.00 I	3.42	0.30
5.00	0.50		
<i>2. Morphine Hydrochloride</i>			
10.65	0.15	4.61	0.48
7.91	0.15	4.11	0.60
6.70	1.00 I	3.86	0.45
5.99	1.00 II	3.57	0.30
5.68	0.60 III	3.38	0.50
4.79	0.30		
<i>3. Morphine Sulfate Dihydrate</i>			
14.69	0.10	3.84	0.20
7.99	0.08	3.53	0.25
7.20	0.50 II	3.41	0.25
6.64	0.13	3.19	0.05
6.24	1.00 I	3.10	0.08
5.47	0.20	2.96	0.02
5.19	0.10	2.86	0.04
4.97	0.15	2.71	0.02
4.69	0.10	2.64	0.04
4.27	0.30 III	2.50	0.00
<i>4. Morphine Sulfate Pentahydrate</i>			
15.10	0.35	4.36	0.80 II
8.35	0.10	4.06	0.05
7.65	0.15	3.86	0.05
7.16	0.15	3.69	0.05
6.21	1.00 I	3.39	0.45
5.64	0.50 III	3.26	0.05
5.24	0.10	3.12	0.15
5.01	0.20	2.63	0.15
4.70	0.05		
<i>5. Morphine Sulfate, Probably Anhydrous</i> (The pentahydrate was heated 185 to 195° C. for two hours)			
15.15	0.5	4.26	0.5
13.46	0.7 III	3.98	0.2
7.91	0.3	3.61	0.2
7.25	0.1	3.41	0.1
6.30	1.0 I	3.35	0.1
5.86	0.6	3.22	0.1
5.51	0.2	3.14	0.1
5.09	0.8 II	2.94	0.2
4.71	0.1	2.70	0.2
4.55	0.1	2.47	0.1
		2.32	0.1
<i>6. Codeine Base</i>			
6.53	1.00 I	3.49	0.10
6.04	0.10	3.23	0.10
5.23	0.33	3.02	0.10
4.89	0.46 II	2.85	0.10
3.89	0.22	2.70	0.10
3.75	0.45 III		
<i>7. Codeine Hydrochloride</i>			
10.33	0.50	3.91	1.00 I
8.04	0.80 III	3.75	0.20
6.11	0.71	3.29	0.20
5.70	0.90 II	3.09	0.20
5.15	0.30	2.97	0.20
4.71	0.25 B	2.77	0.20

TABLE I—CONT'D

d A.U.	I	d A.U.	I
<i>8. Dilaudid (Dihydromorphinone. May contain some NH<sub>4</sub>Cl.)</i>			
10.41	0.29	4.59	0.58 B
9.62	0.40	4.43	
7.15	1.00 I	4.15	
6.62	0.75 II	3.78	0.20
6.03	0.60 III	3.58	0.15
5.54	0.30	3.41	0.48
5.15	0.15	2.64	0.20
4.83	0.10		0.10
<i>9. Dilaudid Hydrochloride</i>			
9.50	0.10	4.36	0.20
6.50	1.00 I	4.07	0.50
6.04	1.00 II	3.50	0.50
5.56	0.50 III	3.37	0.50
5.01	0.20	3.02	0.15
4.72	0.15	2.69	0.25
4.51	0.50		
<i>10. Heroin Base (Diacetylmorphine)</i>			
7.95	0.75 III	4.56	0.25
7.48		4.30	0.60
6.99	0.80 II	3.85	0.40
5.51	1.00 I	3.53	0.50
4.96	0.30 B	3.22	0.20
<i>11. Heroin Hydrochloride</i>			
18.35	0.17	4.63	0.20
9.19	0.70	4.47	0.20
8.04	0.40	4.26	0.30 B
7.18	1.00 I	4.01	0.70
6.84	0.15	3.57	0.30
6.38	0.75	3.36	0.50
5.79	0.90 II	3.18	0.25
5.33	0.20	3.01	0.10
5.13	0.90 III	2.63	0.10
4.91	0.20	2.37	0.10
<i>12. Monoacetylmorphine Hydrochloride</i>			
10.97	0.15	4.34	0.26
8.37	1.00 I	4.10	0.30
6.61	0.50	3.99	
6.11	0.60 II	3.59	0.50
5.92	0.60 III	3.34	0.15
6.62	0.10	3.21	0.25
5.30	0.15	3.05	0.20
5.06	0.40	2.93	0.10
4.70	0.35	2.81	0.10
<i>13. Metopon (Methyldihydromorphinone hydrochloride)</i>			
6.90	1.00 I	3.80	0.20
6.45	0.90 II	3.46	0.30
6.02	0.70	3.15	0.10
5.56	0.30	2.92	0.10
5.10	0.30	2.77	0.10
4.67	0.50	2.71	0.10
4.43	0.20		
4.17	0.85 III		
<i>14. Demerol (Hydrochloride of 1-methyl-4-phenyl-piperidine-4-carboxylic acid ethyl ester)</i>			
7.79	1.00 I	3.70	0.12
6.78	0.22	3.53	0.80 III
5.61	0.70	3.35	0.10
5.16	0.80 II	3.26	0.10
5.03		3.00	0.10
4.59	0.62	2.81	0.10
3.99	0.50	2.54	0.10

After the numbers, I, II, and III represent the three strongest lines in order of their intensity; B indicates an unusually broad line; and brackets are used to indicate two lines which are barely resolved.

limited to alkaloids; any crystalline material may be so identified. Patterns of a number of alkaloids have been prepared by one of us (S. T. G.), and the topochemical method of preparing salts, or the free bases, works quite as well as with the opium alkaloids shown in Table I. Such identifications are of definite toxicologic interest, since the material used is not consumed, the identity is certain, and the diffraction patterns serve as permanent records.

It is possible to obtain diffraction patterns with extremely small amounts of material by using a small sample holder and a very thin powdered sample. Even smaller samples may be used if some inert material, comparatively transparent to the radiation, is added to support the alkaloid. With such a mixture the morphine pattern has been obtained with as little as 0.0007 mg. (0.7  $\gamma$ ) of morphine in the sample. Only the strongest lines are present in patterns containing such small amounts of alkaloid (Fig. 3B). It is necessary that the mounted sample contain at least 1 per cent of morphine; hence, overdilution with the inert material must be avoided. Such a sample was prepared by mixing the free alkaloid in an aqueous suspension with a weighed amount of magnesium oxide to give a thorough dispersion of the insoluble alkaloid. After drying, a small amount of the weighed mixture was used for the sample mount. Reaction between the magnesium oxide and water is quite negligible.

Experiments were carried out by precipitating the base with ammonium hydroxide fumes from a solution of an alkaloidal salt in the presence of a suspension of magnesium oxide. This method is also satisfactory, but diffraction lines from addition products, which must be recognized and discounted, complicate the pattern.

By addition of some known material in a definite proportion to a sample, it is possible to test for the alkaloid quantitatively. Such a quantitative micro-estimation requires a comparison of line intensities and a correction factor. As a description of this method has been published,<sup>4</sup> details will not be given here. The flat cassette type of pattern is suitable for such an analysis, which makes it possible to determine all important constituents of a mixture from one diffraction pattern, provided the necessary reference data are available.

Advantages of the x-ray diffraction technique over other methods of identification are: (1) characteristic and specific diffraction patterns are obtained for any given substance, (2) employment of extremely small amounts of material is possible, (3) a permanent record is obtained, (4) the sample is not destroyed, (5) samples need not be pure (only a small percentage of the material being necessary for identification in the absence of heavy absorbing inorganic salts), (6) all of the materials may be identified in mixtures, although the analysis is more tedious than in the case of a simple compound, and (7) quantitative estimation is possible with a very small change in procedure. Among the disadvantages of this technique, mention should be made of the fact that the presence of either solid solutions or colloids may give unsatisfactory results; however, this condition was not encountered with the materials used in the present study. It is necessary to have standard patterns, or data such as are given in this paper, to carry out the identification.

## SUMMARY

A micromethod for a conclusive identification of various alkaloids, either singly or in mixtures, is described. The specific data necessary for identification of a number of the opium alkaloids are listed in a suitable form for general usage. The sensitivity of the method has been shown to be sufficient to detect and identify quantities of alkaloid as small as 0.0007 mg. (0.7  $\gamma$ ).

A method is described for converting the free base of an alkaloid to a salt in the sample holder without loss of material, whereby two diffraction patterns, rather than one, are used to make identification conclusive.

The authors wish to acknowledge their indebtedness to Dr. Lyndon F. Small, for supplying many of the opiates studied, and to Dr. Howard L. Andrews and Dr. Small, for reading the manuscript and offering helpful suggestions.

## REFERENCES

1. Hanawalt, J. D., Rinn, H. W., and Frevel, L. K.: Chemical Analysis by X-ray Diffraction. *Indust. & Engin. Chem. (Anal. Ed.)* 10: 457, 1938.
2. Oberst, F. W.: The Determination of Morphine in the Urine of Morphine Addicts, *J. LAB. & CLIN. MED.* 24: 318, 1938.
3. Oberst, F. W.: Studies on the Fate of Heroin, *J. Pharmacol. & Exper. Therap.* 79: 266, 1943.
4. Gross, S. T., and Martin, D. E.: Quantitative Determination of Crystalline Materials by X-ray Diffraction, *Indust. & Engin. Chem. (Anal. Ed.)* 16: 95, 1944.
5. Andrews, H. L.: Personal communication.

# A METHOD OF BONE MARROW BIOPSY FROM THE RAT\*

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**I**N STUDIES of experimental anemia it is frequently essential to observe cytologic changes which occur within the bone marrow. In connection with a recent study of the effects of synthetic folic acid (folvite) on certain experimentally induced anemias, some procedure which permitted frequent sampling of the bone marrow in individual rats was essential. Since this procedure has proved most satisfactory and since I am aware of but one published method‡ for obtaining specimens of bone marrow from small animals, I believe that a report of this technique may be of interest to hematologists.

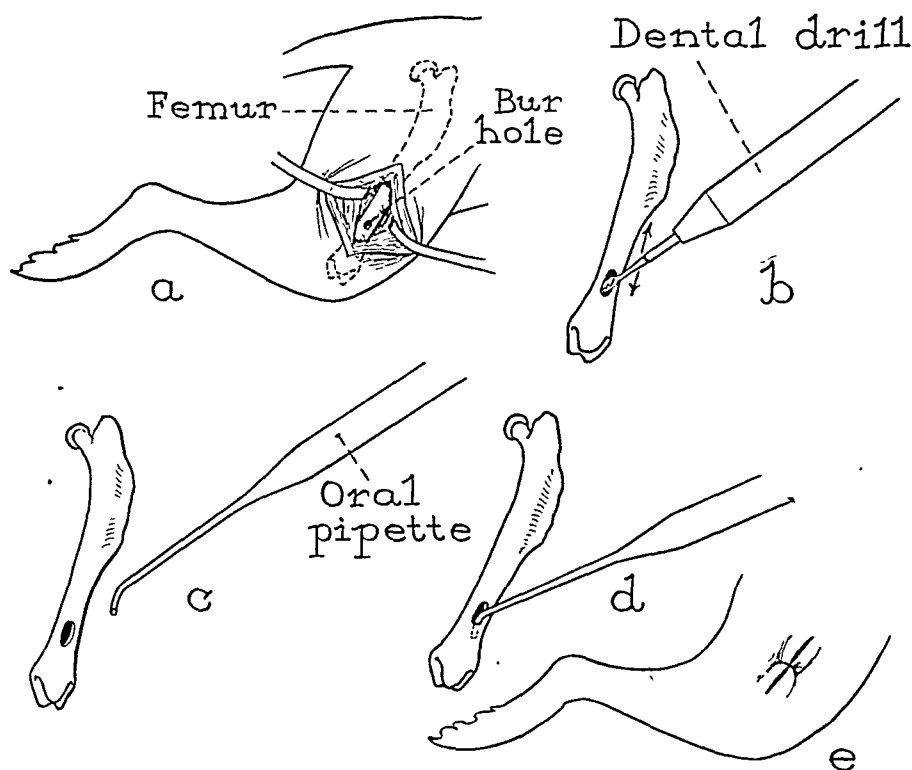


Fig. 1.—Technique of obtaining bone marrow. *a*, Incision of skin and fascia, separation of fibers of biceps, and opening in femur. *b*, Enlargement of opening. *c*, Pipette. *d*, Insertion of pipette. *e*, Closure of incision.

The procedure may be performed quickly, usually requiring less than ten minutes. Anesthesia is induced with an intraperitoneal injection of pentobar-

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\*Work done in the Division of Experimental Medicine, Mayo Foundation, under the direction of Dr. George M. Higgins.

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‡Steinberg, B., and Martin, R. A.: Removal of Bone Marrow in Living Animals, Proc. Soc. Exper. Biol. & Med. 61: 428, 1946.

bital sodium in amounts equal to 30 mg. per kilogram of body weight. Ether anesthesia may also be used. The rat is placed on an operating board in a prone position and the hair over the lateral aspect of the thigh is clipped. An incision approximately  $\frac{1}{2}$  inch (1.3 cm.) in length is made through the skin and fascia directly over the distal third of the femur. The fibers of the short head of the biceps muscle are then carefully separated by means of a small hemostat, thereby exposing the femur. The muscle fibers on either side of the bone are retracted with small aneurysm needles and the periosteum of the femur is stripped away. Using a crosscut dental bur (No. 560), a small opening is made through the cortex into the marrow cavity (Fig. 1a). The circular opening is then slightly enlarged in the direction of the longitudinal axis of the bone, forming an oblong aperture which readily permits the insertion of the tip of an especially designed pipette into the marrow space (Fig. 1b). The pipette, which previously has been rinsed with a solution of heparin, is passed through the opening so that its tip lies in the marrow space parallel to the long axis of the bone (Fig. 1c and d). A small quantity of the marrow is then orally aspirated. Appropriately sized drops are placed on clean glass slides and films are made in the usual manner.

The incision is closed with a single cotton suture extending through the skin into the deep muscles (Fig. 1e). Untoward aftereffects are seldom noted, and the incision is usually well healed within a week. The slides are stained with MacNeal's tetrachrome stain. The procedure may be repeated within a week on the opposite femur and within three weeks on the original one.



## THE USE OF DRIED ANTIGEN PREPARATIONS FOR TESTING PNEUMOCOCCUS TYPING SERA

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THE testing of pneumococcus typing sera for specific and cross reactions according to the requirements of the National Institute of Health served to stimulate the study reported here. The problem was to find a way of preserving pneumococcus typing antigens for a period longer than four days and to demonstrate the successful use of such antigens.

The directions for the preparation of pneumococcus antigens, as given by the National Institute of Health,<sup>1</sup> may be summarized as follows: Each antigen used is a young (one to five hour), fully virulent broth culture to which 1.43 per cent commercial formalin has been added and which has been checked for capsular swelling and agglutination with homologous antiserum. The antigens should be kept in a cold room when not in use and in an iced container when in use. The antigen may be used as long as the pneumococci stay in suspension and the swollen capsules appear intact and are not fragmented, usually a period of three or four days.

The use of dried antigen preparations as a means of preserving pneumococcus antigens for a period longer than four days was selected as feasible and was tested in the following manner: The thirty different types of pneumococcus which were used for such antigens represented strains which had been received from the National Institute of Health, except types 5 and 7 which were obtained from the New York City Department of Health. The antigens were prepared from Felton broth cultures which had been incubated four or five hours at 37° Centigrade. A small loopful (28 gauge platinum loop, 1 mm. inside diameter) of each living antigen was placed on a new clean microscopic slide as in making a Neufeld preparation, and the same sized loopful of purified gastric mucin was mixed well with the antigen but confined to a small area. This was allowed to dry in the air, after which the preparations were stored in regular wooden slide boxes free from dust. Mucin was added as a protective medium for the pneumococci with the idea that the specificity of the dried organisms might be demonstrated over a longer period than otherwise. However, it was found necessary to purify the gastric mucin following the method of Chase,<sup>2</sup> since the crude product contained an amorphous material which interfered with the Neufeld test.

Similar dried preparations were made, using pneumococcus antigens types 1, 2, and 3, which had been formalinized according to the National Institute of Health requirements. In addition, four serial sets of dried living preparations not containing formalin were made for testing cross reactions of pneumococcus typing serum.

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As shown in Table I, the thirty different types of dried pneumococcus antigens prepared with purified gastric mucin gave very good Neufeld reactions for a period of four weeks. Positive agglutination of all the different types of pneumococcus was demonstrated throughout the entire observation period of nine weeks, whereas the capsules gradually disintegrated after four weeks. The pneumococcus antigens which were formalinized, that is, types 1, 2; and 3, gave results which were comparable to those of the dried living antigens.

TABLE I. DURATION OF SPECIFICITY OF DRIED PREPARATIONS OF PNEUMOCOCCUS AS DETERMINED BY NEUFELD REACTION.

TYPES	4/20/39	4/26/39	4/28/39	5/4/39	5/10/39	5/19/39	5/25/39	6/1/39	6/8/39	6/16/39
<i>Dried, Living*</i>										
1	++	++	++	++	++	++	++	++	++	++
2	++	++	++	++	++	++	++	++	++	++
3	++	++	++	++	++	++	++	++	++	++
4	++	++	++	++	++	++	++	++	++	++
5	++	++	++	++	++	++	++	++	++	++
6	++	++	++	++	++	++	++	++	++	++
7	++	++	++	++	++	++	++	++	++	++
8	++	++	++	++	++	++	++	++	++	++
9	++	++	++	++	++	++	++	++	++	++
10	++	++	++	++	++	++	++	++	++	++
11	++	++	++	++	++	++	++	++	++	++
12	++	++	++	++	++	++	++	++	++	++
13	++	++	++	++	++	++	++	++	++	++
14	++	++	++	++	++	++	++	++	++	++
15	++	++	++	++	++	++	++	++	++	++
16	++	++	++	++	++	++	++	++	++	++
17	++	++	++	++	++	++	++	++	++	++
18	++	++	++	++	++	++	++	++	++	++
19	++	++	++	++	++	++	++	++	++	++
20	++	++	++	++	++	++	++	++	++	++
21	++	++	++	++	++	++	++	++	++	++
22	++	++	++	++	++	++	++	++	++	++
23	++	++	++	++	++	++	++	++	++	++
24	++	++	++	++	++	++	++	++	++	++
25	++	++	++	++	++	++	++	++	++	++
27	++	++	++	++	++	++	++	++	++	++
28	++	++	++	++	++	++	++	++	++	++
29	++	++	++	++	++	++	++	++	++	++
31	++	++	++	++	++	++	++	++	++	++
32	++	++	++	++	++	++	++	++	++	++
<i>Dried, Formalinized†</i>										
1	++	++	++	++	++	++	++	++	++	++
2	++	++	++	++	++	++	++	++	++	++
3	++	++	++	++	++	++	++	++	++	++

++, Positive agglutination and capsular swelling.

±, Positive agglutination and less than 90 per cent capsulation, with debris present.

+, Positive agglutination and no capsular swelling.

\*Types 1 to 15, inclusive, prepared 4/18/39; remaining types prepared 4/19/39.

†Formalinized according to National Institute of Health requirements, prepared 4/19/39.

In order to check the efficiency and dependability of the dried antigen preparations for testing pneumococcus typing sera, four such sera were tested: first, by the method required by the National Institute of Health, and second, by the same method except for substitution of dried living antigen preparations in place of formalinized antigens in suspension. After the Neufeld tests were prepared they were incubated for one-half hour at 37° C. as required by the National Institute of Health. Upon examination the dried antigen preparations were found to be as clean-cut in appearance as were the other preparations.

In summary, the dried antigen preparations made from fresh living antigens of thirty different types of pneumococcus gave good specificity tests as determined by Neufeld reactions both for capsular swelling and agglutination for a period of four weeks. After four weeks there was a gradual disintegration of the capsules resulting in a deposit of amorphous debris in the preparations. Similarly prepared formalinized antigen preparations of types 1, 2, and 3 pneumococcus gave corresponding results over the same period.

The dried preparations proved to be equally satisfactory for testing anti-pneumococcus typing serum. The results were equivalent to the results obtained by using the same sera and following the directions given by the National Institute of Health.

Although we were not familiar with Pranion's<sup>3</sup> improved technique for pneumococcus typing by the "Quellung" reaction when this study was in progress, it is interesting to comment in passing that the results obtained by Pranion are in agreement with the findings reported here.

It is also of interest in connection with this study to call attention to Eddy's<sup>4</sup> report of a simple procedure for detecting cross reactions in diagnostic anti-pneumococcic serum.

#### REFERENCES

1. Regulations of the National Institute of Health: Minimum Requirements for Pneumococcic Typing Serum, B-2772, Jan. 16, 1939.
2. Chase, M. W.: A Microorganism Decomposing Group-Specific A Substances, J. Bact. 36: 383, 1938.
3. Pranion, A. B.: Improved Technique for Pneumococcus Typing by the "Quellung" Reaction, J. LAB. & CLIN. MED. 26: 1672, 1941.
4. Eddy, B. E.: A Simplified Procedure for Detecting Cross Reactions in Diagnostic Anti-pneumococcic Serum, Pub. Health Rep. 59: 1041-1045, 1944.

## BOOK REVIEWS

**Acetanilid: A Critical Bibliographic Review.** By *Martin Gross, M.D.*, Research Assistant (Assistant Professor), Laboratory of Applied Physiology, Yale University; with an introduction by *Howard W. Haggard, M.D.*, Director, Laboratory of Applied Physiology, Yale University. Hillhouse Press, New Haven, 1946. Price \$3.00. Cloth with 155 pages.

The content of 763 articles and textbook descriptions concerning acetanilid, a drug which is an active ingredient of many proprietary analgesic preparations, has been critically reviewed and presented in monograph form by Dr. Martin Gross. Support for this extensive bibliographic research came from the Institute for the Study of Analgesic and Sedative Drugs, which in turn derives its funds from contributions from the manufacturers of analgesic and sedative drugs. This is the first of a series of similar monographs soon to be published dealing with salicylates, phenacetin, bromides, and antipyrine.

The author has reviewed the widely scattered literature describing the first preparation of acetanilid in 1852 by Gerhart, the discovery of its antipyretic and analgesic properties by Cahn and Hepp in 1886, the ensuing years of its extensive exploitation for reducing fevers and the later development of therapeutic applications of its analgesic properties, the pharmacologic investigations of its mode of action and pathway of metabolic alteration in the body, and finally the beginning of search by David Lester (1943-1944) and others for the answers to problems described in the review. This summary serves, as Dr. Howard Haggard mentions in the foreword of the book, "to indicate the needs for more definite work on unestablished points and will lead to the avoidance of much unnecessary duplication of studies already made." Dr. Gross has included eight figures and twenty-four tables, many of which represent his own organization or graphic presentation of comparative data from the original sources.

The author's mode of handling controversial subjects is best exemplified by his treatment of the problem of the intermediary metabolism of the drug, a problem which he discusses in the light of twelve series of studies. Many widely quoted statements were found to be based on speculation. Concrete evidence was found available in the form of actual compounds identified and fragmentary evidence from studies of hypothetical substances forming methemoglobin.

In the chapter on pharmacology and toxicology, the mechanisms of and the factors affecting the formation of methemoglobin and of sulfhemoglobin are extensively discussed. It is made apparent that therapeutic doses of acetanilid produce little alteration of body functions other than those involved in analgesia and antipyresis. Many studies have been made with doses in the toxic range, and from these there has emerged no description of any characteristic effect, except for the appearance of cyanosis due to the formation of methemoglobin and sulfhemoglobin.

Despite many attempts several problems concerning acetanilid have been answered unsatisfactorily. Two problems are outstanding. No one has, as yet, adequately explained the mechanism by which it relieves pain, nor has the metabolic alteration of the drug been satisfactorily and definitely established.

Nearly one-half of the monograph is devoted to a résumé of 277 cases of acetanilid poisoning which have appeared in the literature. One-half of these cases occurred during the first ten years of its therapeutic use, a period of very extensive, often ill-directed and poorly controlled, clinical trial. At that time the drug was used mainly as an antipyretic and to a lesser extent as a local antiseptic dusting powder for abraded wounds and burns. In the last eighteen years, twenty-eight of twenty-nine cases of toxicity have resulted from the repeated and often prolonged administration of the drug. The only very characteristic sign of toxicity is cyanosis.

For those interested in the pharmacology and toxicology of analgesic drugs, this monograph will be a very useful guide and source of information.

HAROLD F. CHASE, M.D.

**Early Ambulation and Related Procedures in Surgical Management.** By *Daniel J. Leithauer, M.D., F.A.C.S.*, Chief of Surgery, St. Joseph Mercy Hospital, Detroit, Mich. Charles C Thomas, Publisher, Springfield, Ill., 1946. Price \$4.50. Cloth with 221 pages.

In this small monograph the history of early ambulation after operation is thoroughly but briefly discussed. The physiological justification for its use is carefully outlined, and the author's clinical results are tabulated in detail. Many photographs are presented illustrating the exact method recommended for the first termination of bed rest. The need for exercises and the importance of posture while in bed are also well emphasized and discussed.

By early ambulation the author means that the patient be made to walk within a few hours after operation, if possible, and always within the first twenty-four hours. He emphasizes this as a most important feature, particularly in order to avoid pulmonary complications and vascular accidents. The results achieved are described in considerable detail and include a great reduction in many postoperative complications and disability, a rapid restoration of the patient, and a great shortening in the length of hospital stay.

Over 2,000 operations in which early ambulation was employed form the basis of the author's clinical study. It should be noted, however, that the great majority of these operations were simple. There were only ten gastrectomies in the entire group. Although the period of hospital stay averaged just over two days in the case of appendectomy, patients who had had a gastrectomy remained fifteen days. One of the questions always raised in regard to early ambulation is the possibility that solid wound healing may be impaired thereby, particularly in the case of hernia operations. For this reason the data presented by the author on the recurrence rate following herniotomy are of special interest. In 235 patients operated upon, twelve, or 5.1 per cent, were found to have recurred "insofar as it can be determined by careful follow-up." This is not a high but still a substantial percentage. Yet it is not clear whether all of the patients operated upon were actually followed up. If not, obviously the twelve recurrences would add up to a much higher recurrence rate. The author has made a number of interesting technical observations, one of them of the effect of nylon sutures which he used in 100 patients; he found that in five of them difficulties followed, requiring removal of the sutures.

The monograph is well written and will prove of great interest to surgeons and others who have had no experience with early ambulation. It is no adverse criticism to state that the author is an enthusiast on the subject; indeed, enthusiasm is often needed to break down unjustified traditional resistance to change. On the other hand, it should be mentioned that the problem of postoperative care involves many factors other than the posture and movement of the patient. While a few of the related features have been discussed, the author did not sufficiently emphasize the obvious fact that early ambulation alone is not necessarily synonymous with a smooth postoperative convalescence. Moreover, there are certain contraindications to early rising after operation which are mentioned but insufficiently explained in merely thirteen lines of text. A lesser criticism might be the use of the term "laparotomy syndrome," inasmuch as laparotomy actually means an incision into the side of the patient. A more accurate term would have been celiotomy, but obviously this would not include such operations as herniotomy, thyroidectomy, and mastectomy.

The advantages of early termination of bed rest after operation have been repeatedly emphasized ever since they were first pointed out nearly fifty years ago, yet it is still not a universally applied principle in postoperative care. Surgeons, by clinging to past practices, frequently fail to achieve the maximum in efficient surgical convalescence. The present monograph should serve to challenge tradition and stimulate further interest in the subject, not only in surgical disease but in the therapy of any condition involving bed rest.

ROBERT ELMAN, M.D.

# ANAPHYLACTIC SHOCK IN EGG-SENSITIVE INDIVIDUALS FOLLOWING VACCINATION WITH TYPHUS VACCINE

## A STUDY OF THE ANTIGENIC RELATIONSHIP OF EGG AND CHICKEN MEAT ANTIGENS AND TYPHUS VACCINE

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SINCE the fall of 1942, typhus vaccine prepared on chick egg yolk sacs has been used rather extensively in Army personnel as an immunizing agent against epidemic typhus fever.

Sultzberger and Asher,<sup>1</sup> in 1942, reported three cases suggesting serum sickness syndrome including urticaria following vaccination with yellow fever vaccine, a chick embryo preparation. Swartz<sup>2</sup> reported a case of severe constitutional reaction manifested by urticaria, asthma, and collapse, immediately following an injection of yellow fever vaccine. The serum of this patient was positive in passive transfer to multiple allergens, including egg white and chicken meat as well as the typhus vaccine. Selman<sup>3</sup> reported a case of eosinophilia of 78 per cent in the cerebrospinal fluid attributed to typhus vaccine given six days previously. Roth<sup>4</sup> reported nine cases of various allergic manifestations, including urticaria, asthma, and gastrointestinal symptoms developing immediately following an injection of typhus vaccine. One of these nine case reports cited is typical of anaphylactic shock and is quite similar to the cases reported in this paper. Raynolds<sup>5</sup> emphasized the value of obtaining a history of egg sensitivity in all candidates for chick embryo vaccine inoculations. Sprague and Barnard<sup>6</sup> reported two cases of immediate constitutional reactions, one following an injection of yellow fever vaccine and the other following an injection of typhus vaccine.

Plotz, Coulson and Stevens, and Stull (joint investigation)<sup>7</sup> by extensive and complete studies showed that vaccines prepared from embryonic tissues of developing chicks were allergenic and anaphylactogenic. Plotz<sup>7</sup> found that eleven of 150 subjects injected with typhus vaccine became skin sensitive by test with the vaccine and/or extracts of egg white and egg yolk containing 0.01 mg. nitrogen per cubic centimeter. Coulson and Stevens,<sup>7</sup> using the Schultz-Dale test, showed that typhus and encephalomyelitis vaccines were capable of fatally sensitizing guinea pigs and that the principal antigenic constituents of these vaccines were identifiable with antigens of egg white and egg yolk. These showed further, by precipitin tests, that 12 per cent of the protein nitrogen of typhus vaccine was attributed to crystallizable ovalbumin and concluded

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Since this paper was submitted, Rubin has reported a case of angioneurotic edema due to inoculation with typhus vaccine (*J. Allergy* 17: 21, 1946).

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from this that antigens of egg white, other than ovalbumin, were largely responsible for the sensitizing potency of the vaccine. Stull,<sup>7</sup> using cross-neutralization experiments in passive transfer of egg-sensitive sera, showed the presence of egg white and egg yolk antigens in typhus vaccine in such quantities and specificity that typhus vaccine might produce dangerous systemic reactions in egg-sensitive individuals.

Since July, 1944, the immunization routine with typhus vaccine has been two injections of 1 c.c. each at an interval of seven to ten days. A total of 16,838 injections of typhus vaccine were administered to 8,419 military personnel at this post between Aug. 1, 1944, and Aug. 1, 1945. One anaphylactic reaction occurred in this group. The present article is a report of this case and an additional similar case admitted to this hospital from another Army post, following such a reaction and a study of the antigen-antibody relationship by means of neutralization in passive transfer tests (Prausnitz-Küstner reaction) of the sera of the two patients.

#### CASE REPORTS

CASE 1.—T. G., a 20-year-old soldier with a negative family history of allergic disease, gave a history of nausea and vomiting from ingestion of egg and chicken since infancy, the symptoms lasting thirty to sixty minutes. There was no history of diarrhea, urticaria, asthma, or rhinitis.

In December, 1944, this individual was given initial subcutaneous injections of typhus (1 c.c.) and cholera vaccines (0.5 c.c.) at another Army post. Within five minutes, he became extremely dyspneic and cyanotic and collapsed (fifteen minutes after the injection). The pulse was not perceptible and the heart sounds could not be heard. Epinephrine was administered at intervals of a few minutes for a total dosage of 1.5 cubic centimeters. Aminophyllin was administered intravenously and oxygen inhalation was given continuously for

TABLE I. PASSIVE TRANSFER TESTS WITH SERUM OF PATIENT (T. G., CASE 1) WHO DEVELOPED ANAPHYLACTIC SHOCK FOLLOWING SUBCUTANEOUS INJECTION OF 1 C.C. OF TYPHUS VACCINE; SERUM SITES PREPARED WITH 0.1 C.C. OF SERUM AND TESTED 48 HOURS LATER

SERUM DILUTIONS	TEST ANTIGEN	STRENGTH (PNU PER C.C.)	REACTION
Conc.	Typhus vaccine	100	++++
1:10	Typhus vaccine	100	+++
1:100	Typhus vaccine	100	++
1:1000	Typhus vaccine	100	+
1:10,000	Typhus vaccine	100	0
1:100,000	Typhus vaccine	100	0
Conc.	Egg white	100	++++
1:10	Egg white	100	+++
1:100	Egg white	100	++
1:1000	Egg white	100	+
1:10,000	Egg white	100	0
1:100,000	Egg white	100	0
Conc.	Egg yolk	100	+++
1:10	Egg yolk	100	+++
1:100	Egg yolk	100	++
1:1000	Egg yolk	100	+
1:10,000	Egg yolk	100	0
1:100,000	Egg yolk	100	0
Conc.	Chicken meat	100	+++
1:10	Chicken meat	100	+++
1:100	Chicken meat	100	++
1:1000	Chicken meat	100	+
1:10,000	Chicken meat	100	0
1:1,000,000	Chicken meat	100	0

TABLE II. NEUTRALIZATION EXPERIMENTS IN PASSIVE TRANSFER (SERUM OF PATIENT T. G.)—  
 SERUM-ANTIGEN MIXTURE SITES (0.1 C.C.) PREPARED AND TESTED 48 HOURS LATER  
 WITH 0.025 C.C. TYPHUS VACCINE AND EXTRACTS OF EGG WHITE, EGG YOLK,  
 AND CHICKEN MEAT CONTAINING 100 PNU PER C.C.; ALL TESTS  
 CARRIED OUT IN SAME SKIN RECIPIENT AT SAME TIME

SERUM-ANTIGEN MIXTURES	STRENGTH (PNU PER C.C.)	TEST ANTIGEN (PNU PER C.C.)	REACTION
Serum 1:10 and egg white	100	Typhus vaccine	++
	1,000	Typhus vaccine	+-
	100	Egg white	0
	1,000	Egg white	0
	100	Egg yolk	++
	1,000	Egg yolk	++
	100	Chicken meat	0
	1,000	Chicken meat	0
Serum 1:10 and egg yolk	100	Typhus vaccine	+++
	1,000	Typhus vaccine	++
	100	Egg white	+++
	1,000	Egg white	++
	100	Egg yolk	+
	1,000	Egg yolk	0
	100	Chicken meat	+
	1,000	Chicken meat	0
Serum 1:10 and chicken meat	100	Typhus vaccine	+++
	1,000	Typhus vaccine	+++
	100	Egg white	+++
	1,000	Egg white	+++
	100	Egg yolk	++
	1,000	Egg yolk	+
	100	Chicken meat	+
	1,000	Chicken meat	0
Serum 1:10 and typhus vaccine	100	Typhus vaccine	0
	1,000	Typhus vaccine	0
	100	Egg white	++
	1,000	Egg white	+-
	100	Egg yolk	0
	1,000	Egg yolk	0
	100	Chicken meat	0
	1,000	Chicken meat	0
Serum 1:10 and saline		Typhus vaccine	+++
		Egg white	+++
		Egg yolk	++
		Chicken meat	++

two days. Twenty-four hours after the onset, generalized urticaria developed. All symptoms and signs subsided forty-eight hours later. The patient was admitted to this hospital for study.

Intradermal skin tests showed marked reactions (pseudopodia) with extracts of egg white, egg yolk, and chicken meat containing 100 PNU\* per cubic centimeter and typhus vaccine diluted one thousand times. Tests with egg white extracts in strengths of 1 and 10 PNU per cubic centimeter showed slight and moderate reactions, respectively, without pseudopodia. Skin tests with extracts of thirty-five other common pollens, inhalants, and foods were negative. Skin tests with cholera vaccine in a 1:10 dilution was negative (immediate and delayed).

Passive transfer studies showed this patient's serum to be positive to extracts of egg white, egg yolk, chicken meat, and typhus vaccine even in 1:1000 dilution of the sera (Table I). Cross neutralization tests, using the passive transfer technique with egg and chicken antigens, are shown in Table II.

\*PNU, Protein nitrogen unit, 0.00001 mg. of phosphotungstic acid precipitable nitrogen.



CASE 2.—C. J., a 23-year-old soldier with a negative family history of allergic disease, gave a lifelong history of abdominal cramps, nausea, and vomiting five to ten minutes after ingestion of egg, including food containing small quantities of egg, such as pastries, custards, and mayonnaise. During the past five years, he has had wheezing and dyspnea occasionally associated with the gastrointestinal symptoms following ingestion of egg. He could eat chicken with impunity.

In May, 1945, ten minutes after receiving subcutaneous injections of typhus (1 c.c.) and cholera (0.5 c.c.) vaccines, the patient developed a generalized tingling sensation, nausea, vomiting, and dyspnea, and collapsed in fifteen minutes. Generalized urticaria and edema of lips, eyelids, hands, and feet ensued. The radial pulse was not perceptible and the heart sounds were rapid and only faintly heard. The blood pressure was 70 to 100 systolic over 0 to 60 diastolic, the skin was cold, and the patient was in obvious shock. There were no auscultatory asthmatic signs.

Epinephrine (0.5 c.c.) was administered immediately and the patient was wrapped in blankets. Hot water bottles were applied. Five hundred cubic centimeters of blood plasma, containing 0.5 c.c. of epinephrine, were given intravenously and this was repeated. The patient's skin became warm and the severe shock subsided after 250 c.c. of plasma were administered, but the blood pressure remained low and nausea, vomiting, and abdominal pain continued for eighteen hours. The urticaria lasted two days.

A written description of this patient's experience is as follows: "I received an injection of typhus and cholera at the immunization unit on this post at 1400. After approximately ten minutes, I began to have a choking sensation in my throat and a definite wheezing in my chest. There was a tingling, stinging sensation around the hairline of my head, hands, and feet. My first thought was that I was overheated from the sun. After reaching a shady place, the reaction was no different except that I knew I was getting worse. My feet stung so badly I couldn't stand on them. I couldn't get my breath. I could feel my face and hands begin to swell. I ran to the nearest dispensary which was about two and one-half blocks away. I was having terrific abdominal cramps. I vomited three times as they put me on a stretcher and brought me to the observation ward of the hospital in an ambulance. I had to sit up to

TABLE III. PASSIVE TRANSFER TESTS WITH SERUM OF PATIENT (C. J., CASE 2) WHO DEVELOPED ANAPHYLACTIC SHOCK FOLLOWING SUBCUTANEOUS INJECTION OF 1 C.C. OF TYPHUS VACCINE; SERUM SITES PREPARED WITH 0.1 C.C. OF SERUM AND TESTED 48 HOURS LATER

SERUM DILUTIONS	TEST ANTIGEN	STRENGTH (PNU PER C.C.)	REACTION
Conc.	Typhus vaccine	100	+++
1:10	Typhus vaccine	100	+++
1:100	Typhus vaccine	100	++
1:1000	Typhus vaccine	100	0
1:10,000	Typhus vaccine	100	0
1:100,000	Typhus vaccine	100	0
Conc.	Egg white	100	+++
1:10	Egg white	100	+++
1:100	Egg white	100	++
1:1000	Egg white	100	0
1:10,000	Egg white	100	0
1:100,000	Egg white	100	0
Conc.	Egg yolk	100	+++
1:10	Egg yolk	100	++
1:100	Egg yolk	100	++
1:1000	Egg yolk	100	0
1:10,000	Egg yolk	100	0
1:100,000	Egg yolk	100	0
Conc.	Chicken meat	100	+++
1:10	Chicken meat	100	++
1:100	Chicken meat	100	++
1:1000	Chicken meat	100	0
1:10,000	Chicken meat	100	0
1:100,000	Chicken meat	100	0

TABLE IV. NEUTRALIZATION EXPERIMENTS IN PASSIVE TRANSFER (SERUM OF PATIENT C. J.)—  
 SERUM-ANTIGEN MIXTURE SITES (0.1 C.C.) PREPARED AND TESTED 48 HOURS LATER  
 WITH 0.025 C.C. TYPHUS VACCINE AND EXTRACTS OF EGG WHITE, EGG YOLK,  
 AND CHICKEN MEAT CONTAINING 100 PNU PER C.C.; ALL TESTS  
 CARRIED OUT IN SAME SKIN RECIPIENT AT SAME TIME

SERUM-ANTIGEN MIXTURES	STRENGTH (PNU PER C.C.)	TEST ANTIGEN (PNU PER C.C.)	REACTION
Serum 1:10 and egg white	10	Typhus vaccine	++
	100	Typhus vaccine	+-
	1,000	Typhus vaccine	0
	10	Egg white	+-
	100	Egg white	0
	1,000	Egg white	0
	10	Egg yolk	+
	100	Egg yolk	+-
	1,000	Egg yolk	0
	10	Chicken meat	+
	100	Chicken meat	0
	1,000	Chicken meat	0
Serum 1:10 and egg yolk	10	Typhus vaccine	++
	100	Typhus vaccine	+
	1,000	Typhus vaccine	+-
	10	Egg white	++
	100	Egg white	+
	1,000	Egg white	+-
	10	Egg yolk	+
	100	Egg yolk	0
	1,000	Egg yolk	0
	10	Chicken meat	+
	100	Chicken meat	+-
	1,000	Chicken meat	0
Serum 1:10 and chicken meat	10	Typhus vaccine	++
	100	Typhus vaccine	++
	1,000	Typhus vaccine	+
	10	Egg white	+
	100	Egg white	+-
	1,000	Egg white	0
	10	Egg yolk	+
	100	Egg yolk	0
	1,000	Egg yolk	0
	10	Chicken meat	+
	100	Chicken meat	0
	1,000	Chicken meat	0
Serum 1:10 and typhus vaccine	10	Typhus vaccine	+
	100	Typhus vaccine	0
	1,000	Typhus vaccine	0
	10	Egg white	++
	100	Egg white	+
	1,000	Egg white	0
	10	Egg yolk	+-
	100	Egg yolk	0
	1,000	Egg yolk	0
	10	Chicken meat	+-
	100	Chicken meat	0
	1,000	Chicken meat	0
Serum 1:10 and saline		Typhus vaccine	+++
		Egg white	+++
		Egg yolk	++
		Chicken meat	++

get my breath. I fought an oxygen mask. I vomited time after time. My face, hands, and feet became swollen and my feet ached. I was still having a lot of trouble with breathing."

Intradermal skin tests showed marked reactions (pseudopodia) with extracts of egg white, egg yolk, and chicken meat containing 100 PNU per cubic centimeter and typhus vaccine diluted one hundred times. Skin tests with extracts of thirty-five common pollens, inhalants, and foods, in strength of 10,000 PNU per cubic centimeter, were negative. Skin test with cholera vaccine in 1:10 dilution was negative (immediate and delayed reactions).

Passive transfer studies showed this patient's serum to be positive to extracts of egg white, egg yolk, chicken meat, and typhus vaccine in 1:100 dilutions of the serum (Table III). Cross neutralization tests, using the passive transfer technique with extracts of egg white, egg yolk, chicken meat, and typhus vaccine, are shown in Table IV.

#### NEUTRALIZATION EXPERIMENTS IN PASSIVE TRANSFER

Neutralization tests in passive transfer were carried out with the sera of the two patients (Cases T. G. and C. J.) presented. Dilutions of 1:10 of each serum were prepared with normal saline. The diluted sera were mixed with equal quantities of typhus vaccine and extracts of egg white, egg yolk, and chicken meat in varying strengths. Skin sites were prepared with 0.1 c.c. of each mixture in a recipient reacting negatively to test with the most concentrated form of each material used. The serum-antigen mixture skin sites were tested forty-eight hours later with typhus vaccine and with extracts of egg white, egg yolk, and chicken meat containing 100 PNU per cubic centimeter (Tables II and IV).

The serum of one patient (T. G. Case 1) was significantly neutralized to test with typhus vaccine by extracts of egg white, only negatively by extracts of egg yolk and not at all by extract of chicken meat in the strength of extracts used. Typhus vaccine, on the other hand, neutralized completely to test with extracts of egg yolk and chicken meat but only partially to the extract of egg white. Egg white and egg yolk neutralized to test with each other only negligibly. Egg white and egg yolk completely neutralized to test with chicken meat, although it required a strength of 1,000 PNU per cubic centimeter in the case of egg yolk. Chicken meat failed to neutralize significantly to test with either egg white or egg yolk (Table II). The experiment with serum of this patient was repeated using another skin recipient, and essentially the same results were obtained.

The serum of another patient (C. J., Case 2) was significantly neutralized by the respective antigens similarly as in the case of serum of patient T. G., except that less concentration of antigens was needed to neutralize the serum (Table IV). In addition, egg yolk and chicken meat, although failing to neutralize completely to test with typhus vaccine, did show definite neutralizing qualities, more so than in the experiments with serum of patient T. G. A greater tendency toward cross neutralization by the egg white, egg yolk, and chicken meat was noted with serum of patient C. J. than with that of patient T. G. (Tables II and IV). The experiment with serum of patient C. J. was repeated twice, using different skin recipients, and essentially the same results were obtained.

The fact that each antigen neutralized to test with itself indicated that the strength of extracts used in the experiments were in proper range.

## EXHAUSTION NEUTRALIZATION TESTS IN PASSIVE TRANSFER

At the time the two sera were retested in determining the quantity of skin-sensitizing antibody contained therein (the dilution at which the serum no longer reacted in passive transfer), serum sites were prepared with 1:100 and 1:1000 dilutions of each serum. It was confirmed that serum of patient T. G. was positive in the 1:1000 dilutions, whereas, serum of patient C. J. was positive in the 1:100 but negative in the 1:1000 dilutions when tested with egg white, egg yolk, chicken meat, and typhus vaccine.

These prepared serum sites were retested at one- or two-day intervals with the original testing antigen until no reaction occurred. The sites were then

TABLE V. EXHAUSTION NEUTRALIZATION TESTS IN PASSIVE TRANSFER (SERUM OF PATIENT T. G.)—SKIN SITES PREPARED WITH 0.1 C.C. SERUM; ALL TESTS MADE WITH EXTRACT CONTAINING 100 PNU PER C.C.

10/11/45	10/12/45		10/13/45		10/15/45		10/16/45	
SERUM DILUTIONS	TEST ANTIGEN	REACTION	TEST ANTIGEN	REACTION	TEST ANTIGEN	REACTION	TEST ANTIGEN	REACTION
1:100	Chicken meat	++	Chicken meat	+-	Chicken meat	0	Typhus vaccine	+
1:1000	Chicken meat	+	Chicken meat	0	Chicken meat	0	Typhus vaccine	0
1:100	Egg white	++	Egg white	0	Typhus vaccine	+-		
1:1000	Egg white	+	Egg white	0	Typhus vaccine	0		
1:100	Egg yolk	++	Egg yolk	+	Egg yolk	0	Typhus vaccine	+
1:1000	Egg yolk	+	Egg yolk	+-	Egg yolk	0	Typhus vaccine	0
1:100	Typhus vaccine	++	Typhus vaccine	+	Typhus vaccine	0	Egg white	+
1:1000	Typhus vaccine	+	Typhus vaccine	0	Typhus vaccine	0	Egg white	0

TABLE VI. EXHAUSTION NEUTRALIZATION TESTS IN PASSIVE TRANSFER (SERUM OF PATIENT C. J.)—SKIN SITES PREPARED WITH 0.1 C.C. SERUM; ALL TESTS MADE WITH EXTRACT CONTAINING 100 PNU PER C.C.

10/11/45	10/12/45		10/13/45		10/15/45		10/16/45	
SERUM DILUTIONS	TEST ANTIGEN	REACTION	TEST ANTIGEN	REACTION	TEST ANTIGEN	REACTION	TEST ANTIGEN	REACTION
1:100	Chicken meat	+	Chicken meat	+-	Chicken meat	0	Typhus vaccine	0
1:1000	Chicken meat	0	Chicken meat	0	Chicken meat	0	Typhus vaccine	0
1:100	Egg white	++	Egg white	+-	Egg white	0	Typhus vaccine	+-
1:1000	Egg white	0	Egg white	0	Egg white	0	Typhus vaccine	0
1:100	Egg yolk	++	Egg yolk	+-	Egg yolk	0	Typhus vaccine	0
1:1000	Egg yolk	0	Egg yolk	0	Egg yolk	0	Typhus vaccine	0
1:100	Typhus vaccine	++	Typhus vaccine	+	Typhus vaccine	0	Egg yolk	0
1:1000	Typhus vaccine	0	Typhus vaccine	0	Typhus vaccine	0	Egg yolk	0

tested with typhus vaccine. Extracts of egg white and egg yolk were used when typhus vaccine was the original testing antigen.

In the case of serum of patient T. G., typhus vaccine induced a slightly positive reaction after the 1:100 dilution serum sites were exhausted with chicken meat and egg yolk extracts (100 PNU per cubic centimeter), respectively, but failed to do so in the 1:1000 dilution sites. The typhus vaccine reaction was questionable in the 1:100 dilution site exhausted by egg white. A slight reaction was induced by egg white in the 1:100 dilution site exhausted by typhus vaccine (Table V).

In the case of serum of patient C. J., typhus vaccine induced a questionable reaction after the 1:100 dilution serum site was exhausted with egg white extract (100 PNU per cubic centimeter). The remaining reactions were negative as shown in Table VI.

#### PRECIPITIN TESTS

Precipitin ring tests were carried out with the concentrated sera (Patients T. G. and C. J.) and serial dilutions of typhus vaccine from 1:2 to 1:512. No precipitins were demonstrable.

#### COMMENT

The case reports of anaphylactic shock following parenteral injections of typhus vaccine prepared on chick embryo yolk sacs presented and the demonstration of positive skin tests and skin sensitizing antibodies in the serum of these patients to typhus vaccine indicate that typhus vaccine may be antigenic and dangerous when injected into hypersensitive individuals.

The demonstration of positive skin tests to and skin-sensitizing antibodies in the sera of typhus vaccine-sensitive patients to egg white, egg yolk, and chicken meat suggests a close antigenic relationship between typhus vaccine and these antigens.

The cross neutralization experiments described with typhus vaccine by egg white, egg yolk, and chicken meat antigens in passive transfer studies strongly indicate the active antigenic factors in typhus vaccine to be egg white, egg yolk, and chicken meat; the egg white is probably the most important, and the latter two, respectively, are less important. This confirms the work of Stull.<sup>7</sup>

The exhaustion neutralization experiments further indicate that the principle antigenic factors in typhus vaccine are those of egg white, egg yolk, and chicken meat but that there may be an additional one unrelated to these antigens. If the latter is true, such antigen is probably present in minute quantity since the reactions obtained with typhus vaccine in serum sites exhausted by egg and chicken antigens were of small magnitude.

It is suggested that all candidates for injections of typhus vaccine or other chick embryo vaccines be thoroughly questioned before such injections are administered and that all probable egg sensitive individuals be skin tested intradermally with approximately 0.02 c.c. of typhus vaccine diluted with normal saline to 1:100 and, if indicated, 1:10 dilution before vaccination is instituted.

It is recommended that individuals showing positive skin reactions to typhus vaccine and other similar chick embryo vaccines be exempt from vaccination with these materials.

Whereas minor reactions not coming to the attention of the medical personnel may have occurred, the fact that only one anaphylactic reaction occurred in 8,419 individuals receiving typhus vaccine, coupled with the fact that egg sensitivity has been considered one of the most common of all food sensitivities, suggests a low incidence of food allergy in military personnel, as previously reported.<sup>8</sup>

#### SUMMARY

1. Two cases of anaphylactic shock following parenteral injections of *typhus vaccine* prepared from chick embryos infected with epidemic typhus rickettsia are reported.

2. Intradermal skin tests, passive transfer tests, and neutralization experiments with serum from the two cases presented indicated a likely antigenic identity of typhus vaccine and the egg and chicken meat antigens contained therein.

3. It is recommended that all candidates for typhus vaccine injections or other similar chick embryo vaccines be questioned for possible egg sensitivity and that all probable egg-sensitive individuals be skin tested with typhus vaccine prior to parenteral administration thereof. A suggested initial testing strength is a 1:100 dilution of the vaccine using approximately 1/50 c.c. volume. These precautionary measures have been advised by the War Department TB MED 114, Nov. 9, 1944.

4. Since *influenza virus vaccine* (types a and b) is prepared from infected chick embryos, it is suggested that the same precautions be taken with this vaccine.

Acknowledgment is made of the valuable assistance of Sergeant Jack Ladwig, Sergeant Walter Goodey, and Corporal David Mitzkus.

#### REFERENCES

1. Sulzberger, M. B., and Asher, C.: Urticaria and Erythema Multiforme-Like Eruptions Following Injections of Yellow Fever Vaccine, U. S. Nav. M. Bull. 40: 411, 1942.
2. Swartz, H.: Systemic Allergic Reaction Induced by Yellow Fever Vaccine, J. LAB. & CLIN. MED. 28: 1663, 1943.
3. Selman, D.: Eosinophilia in Cerebrospinal Fluid, Bull. U. S. Army M. Dept. 86: 121, 1945.
4. Roth, V. E.: Reactions to Typhus Vaccine, Bull. U. S. Army M. Dept. 88: 111, 1945.
5. Raynolds, A. H.: Immunization With Multiple Antigens, J. A. M. A. 128: 613, 1945.
6. Sprague, H. B., and Barnard, J. H.: Egg Allergy; Significance in Typhus and Yellow Fever Immunization, U. S. Nav. M. Bull. 45: 71, 1945.
7. Allergic and Anaphylactogenic Properties of Vaccines Prepared From Embryonic Tissues of Developing Chicks: A Joint Investigation Initiated by the Division of Virus and Rickettsial Diseases, Army Medical School, Washington, D. C.
  - (I) Plotz, H.: Skin Sensitivity Following the Subcutaneous Inoculation of Typhus Vaccines in Man, J. Immunol. 53: 315, 1946.
  - (II) Coulson, E. J., and Stevens, H.: Anaphylactogenic Properties of Typhus Fever Vaccines and Equine Encephalomyelitis Vaccines, Ibid. p. 321.
  - (III) Stull, A.: A Study to Determine Whether Chick-Embryo Yolk Sac Vaccines Contain Sufficient Egg Proteins to Cause Severe Systemic Reactions If Given to Egg-Sensitive Individuals, Ibid. p. 343.
8. Hampton, S. F., and Rand, H.: The Problem of Allergy at an Army Air Forces Hospital, J. Allergy 15: 355, 1944.

## THE RELATIONSHIP OF GASTRIC ACIDITY TO THIAMINE EXCRETION IN THE AGED

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**I**N A study of the gastric secretory response in the aged, it was shown that while hyperchlorhydria was not infrequently found the majority of the subjects had 19 degrees or less of free hydrochloric acid in the gastric juice.<sup>1</sup> In previous articles<sup>2-4</sup> it was also demonstrated that there is a tendency to avitaminosis in the aged. Jolliffe<sup>5</sup> suggested that the failure of some elderly people to absorb thiamine may be due to the achlorhydria so frequently encountered in this age group.

The relationship between gastric acidity and secretion and thiamine has been extensively studied. Notwithstanding the numerous references on this subject, however, a difference of opinion still exists as to the effect of thiamine deficiency upon the gastric acidity and secretion. As a result of the work of Cowgill and Gilman on dogs,<sup>6</sup> the prevailing opinion has been that thiamine deficiency suppresses the gastric secretion. Joffe and Jolliffe, in 1937, postulated the presence of an achlorhydria-preventive factor in the vitamin B complex, which was not identical with either vitamin B<sub>1</sub> or the antipellagra factor.<sup>7</sup> Danysz-Michel and Koskonski<sup>8</sup> found a decrease in volume, acidity, and peptic activity of the gastric secretion elicited by histamine in a study of pigeons kept on a diet of polished rice. Gildea and associates<sup>9</sup> were unable to find any change in the acidity or the pepsin concentration of the gastric secretion evoked by histamine in dogs kept on Cowgill's Casein III diet. Shay and co-workers<sup>10</sup> recently reported their results of the effect of thiamine chloride depletion upon the spontaneous gastric secretion in rats. They found that after an acute severe thiamine depletion the volume of spontaneous gastric secretion was much greater than in their corresponding controls, while the acidity and peptic power remained normal. Elsom<sup>11</sup> kept two human beings on a diet deficient in thiamine; he found that the gastric acidity was normal after an oatmeal gruel meal in one subject and that a normal gastric secretory response to histamine was obtained in the other. Williams and associates<sup>12</sup> reported a low level or an absence of acidity in four young women maintained on a low thiamine diet.

In view of the differences of opinions as to the effects of thiamine deficiency upon the gastric acidity and secretion and in view of the tendency of aged individuals toward a decreased gastric acidity, as well as the prevalence of avitaminoses in elderly subjects, it was thought of interest to study the relationship between the thiamine levels and the gastric acidity in a group of apparently normal individuals 65 years of age and over.

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## EXPERIMENTAL PROCEDURE

This study is based on a series of thirty-one persons whose ages ranged from 65 to 81 years. Fourteen were men and seventeen were women. All were apparently normal individuals who were ambulatory and who were fed the routine institutional diet which was adequate in thiamine content. A chemical analysis showed that this diet contained per day from 0.51 to 1.11 mg. of thiamine, 0.97 to 1.78 mg. of riboflavin, 16.0 to 117 mg. of ascorbic acid, 2.5 to 10.0 mg. of iron, 6.9 to 13.1 mg. of nicotinic acid, 1,500 to 11,800 U. S. P. units of carotene, and from 1,300 to 5,500 U. S. P. units of vitamin A.\* The results of the analyses of six separate twenty-four hour periods may be seen in Table I. The methods used for the vitamin analyses were those of Hennessey and Cerecedo<sup>13</sup> for the thiamine, Rubin and co-workers<sup>14</sup> for the ascorbic acid, the American Association of Cereal Chemists<sup>16a</sup> for the iron, Krehl and associates<sup>16b</sup> for the nicotinic acid, the Association of Official Agricultural Chemists<sup>17</sup> for the carotene, and Dann and Evelyn<sup>18</sup> for the Vitamin A.

TABLE I. VITAMIN ASSAY PER TWENTY-FOUR HOUR PERIOD

TWENTY-FOUR HOUR PERIOD	METHOD						
	B <sub>1</sub> <sup>13</sup>	B <sub>2</sub> <sup>14</sup>	C <sup>15</sup>	IRON <sup>16a</sup>	NIACIN <sup>16b</sup>	CAROTENE <sup>17</sup>	VITAMIN A <sup>18</sup>
First	0.88	0.97	59	5.6	11.6	6,000	2,700
Second	0.83	1.33	16	10.0	10.4	11,400	1,900
Third	1.11	1.78	117	3.7	13.1	11,800	1,900
Fourth	0.84	1.22	16	9.0	6.9	2,100	1,300
Fifth	0.70	1.29	18	2.5	8.6	8,800	1,300
Sixth	0.51	1.03	40	10.9	10.6	1,500	5,500

Results are expressed in milligrams per day, except carotene and vitamin A which are U. S. P. units per day.

The method of procedure was as follows: Twenty-four hour specimens of urine, collected under toluene and stored in the refrigerator, were analyzed for their thiamine content.<sup>13</sup> An analysis of the gastric contents with the subject in the fasting state, as well as the secretory response after a water and toast test meal, was made. These determinations were done fractionally. An attempt was made to extend the analyses for one hour, but this was not always feasible in elderly subjects. If no free hydrochloric acid was found, the subjects were given an injection of histamine hydrochloride in the dosage of 0.1 mg. per 10 kilograms of body weight. These determinations were carried out for one hour.

## RESULTS

In Fig. 1 there are plotted the thiamine chloride levels and the determinations of the fasting-free hydrochloric acid, and in Fig. 2 are plotted the B<sub>1</sub> levels when the free hydrochloric acid was at its height. The experimental data may be seen in Table II.

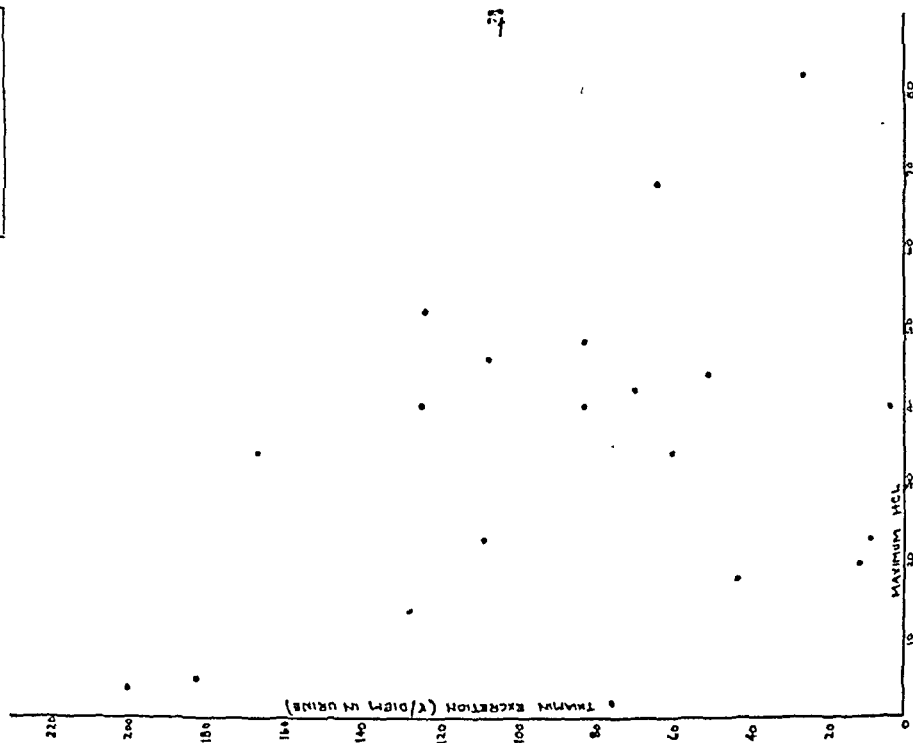
A thiamine excretory level below 50  $\mu$ g per day was regarded as subnormal.<sup>19</sup>

Of the thirty-one subjects studied, fourteen, or 45.1 per cent, had low hydrochloric acid values. Six had true achlorhydria, that is, no free hydro-

\*The vitamin assays of the diets were performed by Dr. S. H. Rubin of Hoffmann-La Roche, Inc., Nutley, N. J.



RELATIONSHIP BETWEEN  
URINARY THIAMIN EXCRETION  
AND MAXIMUM HCL



RELATIONSHIP BETWEEN  
FASTING FREE HCL AND  
URINARY THIAMIN EXCRETION

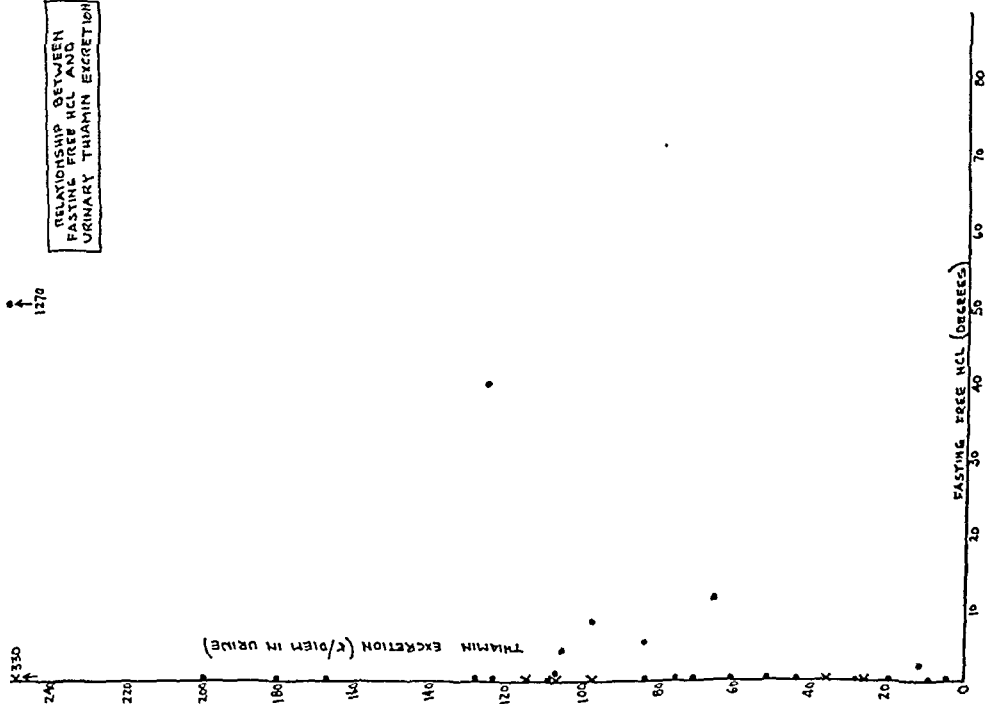


TABLE II

NAME	SEX	AGE	FASTING HCl	FASTING TOTAL	MAXIMUM HCl	MAXIMUM TOTAL	URINARY THIAMINE ( $\mu$ G./DAY)
H. G.	M	70	3	16	52	71	492
J. B.	M	68	8	14	Not done		97
C. M.	M	78	0	1	2	11	76
M. M.	F	74	1	6	46	68	108
J. K.	M	70	2	8	20	51	12
R. L.	F	80	0	5	18	66	44
S. D.	F	80	0	15	42	64	71
S. S.	F	78	50	68	50	68	1070
L. M.	F	65	0	10	40	60	84
A. K.	M	74	0	8	34	60	167
A. A.	M	76	0	40	34	64	61
M. H.	F	75	0	8	5	18	181
L. F.	M	78	0	30	52	82	124
S. N.	M	71	0	20	40	66	4
J. D.	M	78	4	8	98	108	106
D. S.	M	78	11	26	68	86	66
L. L.	F	79	0	14	82	102	28
B. B.	F	73	0	10	23	43	109
I. F.	F	81	5	11	48	64	84
J. S.	M	71	0	8	44	64	52
S. J.	M	77	40	54	40	54	125
L. T.	M	80	0	9	40	56	Not done
E. D.	F	69	0	10	23	55	9
M. A.	F	73	0	4	4	26	200
D. L.	F	79	0	2	14	35	128
<i>The Following Subjects Had True Achlorhydria:</i>							
R. G.	F	76	0	13	0	28	36
R. F.	F	70	0	10	0	13	330
F. K.	F	70	0	18	0	18	26
L. H.	M	67	0	8	0	25	115
B. B.	F	80	0	13	0	13	108
A. B.	F	73	0	8	0	8	98

chloric acid was obtained after the injection of histamine. Two of the six subjects had subnormal daily urinary excretions of thiamine, and four had levels ranging from 98 to 330  $\mu$ g per day. Nineteen subjects had from 2 to 49 degrees of free hydrochloric acid, and ten of these showed abnormally low thiamine excretion values. Six of the thirty-one subjects had 50 degrees or more of free hydrochloric acid after a water and toast test meal. Two of these six subjects showed subnormal thiamine excretions. One had a free hydrochloric acid of 82 and a total acidity of 102 degrees, with a thiamine excretory level of 28  $\mu$ g per day, and the other had a free hydrochloric acid of 68 and a total acidity of 86 degrees, with a daily vitamin B<sub>1</sub> excretion of 66 micrograms.

## DISCUSSION

As was stated before, a difference of opinion exists as to whether there is any causal relationship between gastric acidity and the thiamine status of an individual. This was of especial interest to us in view of the tendency to hypochlorhydria and avitaminoses in elderly individuals. In this study we found both high and low thiamine values in subjects with anacidity, hypochlorhydria, and hyperchlorhydria. These findings are illustrated in Fig. 2. Since a low

thiamine excretion is compatible with a histamine-fast achlorhydria as well as with a hyperchlorhydria, it is questionable whether there is any causal relationship between the gastric secretory response and thiamine deficiency in apparently normal elderly subjects. In trying to explain the low thiamine levels in the aged, several factors must be taken into account. The most important of these are inadequate intake, lack of assimilation or absorption, and improper utilization of the amount that has been absorbed. In this study the factor of inadequate intake was ruled out by careful vitamin analysis of the diet. Furthermore, as will be shown in a subsequent paper<sup>20</sup> the subjects took a sufficient amount of food which was determined by careful supervision as well as by weighing and measuring their intake. In regard to assimilation or absorption, the "conditioned malnutrition states" as described by Jolliffe<sup>5</sup> were not present. It is our opinion that the tendency to avitaminosis in the aged arises from a lack of proper utilization of the amount that has been absorbed. This subject is still under investigation and will be reported in a forthcoming paper.

In appraising thiamine levels in individuals past the age of 65, another possibility must be kept in mind. The so-called normal levels of the various vitamins that have appeared in the literature are based on studies on infants, children, and young healthy adults. It is possible that these values do not apply to the age group that we have been studying. As an example of just such an occurrence, a series of studies by Newman and Gitlow<sup>21, 22</sup> may be cited. These authors showed that the normal values reported in the literature for as basic a physiologic value as red cell, hemoglobin, and white cell values do not seem to be applicable to individuals past the age of 65. It is conceivable that we may be dealing with the same type of standards in the case of thiamine.

#### SUMMARY AND CONCLUSIONS

1. Of thirty-one elderly subjects studied, 45.1 per cent had low thiamine excretory levels.
2. The fasting gastric acidity and the secretory response after a test meal were determined in all of these subjects.
3. Both high and low excretory levels of thiamine were found in subjects with anacidity, hypochlorhydria, and hyperchlorhydria.

#### REFERENCES

1. Rafsky, H. A., and Weingarten, M.: A Study of the Gastric Secretory Response in the Aged, *Gastroenterology*. In press.
2. Rafsky, H. A., and Newman, B.: Vitamin C Studies in the Aged, *Am. J. M. Sc.* 201: 749, 1941.
3. Rafsky, H. A., and Newman, B.: Relationship of Niacin (Nicotinic Acid), to Porphyrinuria in the Aged, *Am. J. M. Sc.* 205: 209, 1943.
4. Rafsky, H. A., and Newman, B.: Vitamin B<sub>1</sub> Excretion in the Aged, *Gastroenterology* 1: 737, 1943.
5. Jolliffe, N.: Handbook of Nutrition; Conditioned Malnutrition, *J. A. M. A.* 122: 299, 1943.
6. Cowgill, G. R., and Gilman, A.: Physiology of Vitamins; Effect of Lack of the Vitamin B Complex on the Secretion of Gastric Juice in Dogs With Gastric Pouches, *Arch. Int. Med.* 53: 58, 1934.
7. Joffe, P. M., and Jolliffe, N.: The Gastric Acidity in Alcohol Addicts, With Observations on the Relation of the B Vitamins to Achlorhydria, *Am. J. M. Sc.* 193: 501, 1937.

8. Danysz-Michel and Koskonski, W.: Vitamin B, *Compt. rend. Soc. de Biol.* 85: 54, 1922.
9. Gildea, E. F., Kattwinkel, E. E., and Castle, W. B.: Experimental Combined System Disease, *New England J. Med.* 202: 523, 1930.
10. Shay, H., Komarov, S. A., Gruenstein, M., and Fels, S. S.: The Effect of Thiamin Deprivation Upon Gastric Secretion in Rats, *Gastroenterology* 6: 199, 1946.
11. Elsom, K. O.: Experimental Study of Clinical Vitamin B Deficiency, *J. Clin. Investigation* 14: 40, 1935.
12. Williams, R. D., Mason, H. L., and Smith, B. F.: Induced Vitamin B<sub>1</sub> Deficiency in Human Subjects, *Proc. Staff Meet., Mayo Clin.* 14: 787, 1939.
13. Hennessey, D. J., and Cerecedo, L. R.: The Determination of Free and Phosphorylated Thiamin by a Modified Thiochrome Assay, *J. Am. Chem. Soc.* 61: 179, 1939.
14. Rubin, S. H., De Ritter, E., Schuman, R. L., and Bauernfeind, J. C.: The Determination of Riboflavin in Low Potency Foods and Feeds, *Ind. Eng. Chem.* 17: 136, 1945.
15. Robinson, W. B., and Stotz, E.: The Indophenol-xylene Extraction Method for Ascorbic Acid, *J. Biol. Chem.* 160: 217, 1945.
16. (a) A. A. C. C. Method: Determination of Iron in Food Products, ed. 4, Washington, D. C., 1941, p. 47.  
 (b) Krehl, W. A., Strong, F. M., and Elvehjem, C. A.: Determination of Nicotinic Acid, *Ind. Eng. Chem., Anal. Ed.* 15: 471, 1943.
17. A. O. A. C.: Official and Tentative Methods for Analysis, ed. 5, Washington, D. C. 1940, p. 369.
18. Dann, W. J., and Evelyn, K. A.: The Determination of Vitamin A With the Photoelectric Colorimeter, *Biochem. J.* 32: 1008, 1938.
19. Sevringhaus, E.: Personal communication.
20. Rafsky, H. A., and Newman, B. Unpublished data.
21. Newman, B., and Gitlow, S.: Blood Studies in the Aged. I. The Erythrocyte in the Aged Male and Female, *Am. J. M. Sc.* 205: 677, 1943.
22. Newman, B., and Gitlow, S.: Blood Studies in the Aged. II. The Leukocytes in the Aged Male and Female, *Am. J. M. Sc.* 206: 622, 1943.

# THE EVALUATION OF THE VITAMIN C STATUS OF HUMAN SUBJECTS: STUDIES WITH INTRADERMAL DYE DISCOLORATION, CAPILLARY FRAGILITY, FASTING PLASMA VITAMIN C LEVEL, AND VITAMIN C SATURATION

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THE recognition of a subclinical scorbutic state has assumed major importance in recent years. Normal or near normal concentrations of vitamin C in the tissues are necessary for the normal function of the capillary wall, for adequate wound healing and the repair of fractures, and for the normal metabolism of some amino acids. This vitamin, moreover, may play a part in specific enzyme systems and detoxification functions relative to sulfanilamide type compounds. Consequently, emphasis has been placed on various laboratory procedures which might be utilized to demonstrate vitamin C saturation or unsaturation.

In 1938 Rotter<sup>1</sup> described an intradermal dye discoloration test. His technique was tested extensively by a number of investigators including Portnoy and Wilkinson<sup>2</sup> and Suzuki<sup>3</sup> who maintained that a satisfactory correlation existed between this procedure and the blood vitamin C level. Beck and Krieger<sup>4</sup> found this test to agree with the results obtained by vitamin C saturation studies. In a recent paper Slobody<sup>5</sup> found that the skin test denoted the degree of body saturation with vitamin C. He concluded that the discrepancy between plasma ascorbic acid levels and the results of the skin tests could be explained by the fact that the blood level at any given time reflected only the recent intake of vitamin C. This statement requires further confirmation since Slobody showed that the intravenous administration of 200 mg. of vitamin C decreased the intradermal skin test time in forty-four of forty-five cases as the plasma vitamin C level rose; this implies a reciprocal relationship between the two procedures. Goldsmith and associates<sup>6</sup> concluded that no correlation existed between the plasma vitamin C level of fasting subjects and intradermal dye discoloration. Wright and MacLenathen<sup>7</sup> observed that the variation in the discoloration time using Rotter's method was too great to establish a normal range; moreover, these workers showed that discoloration times varied appreciably at different body sites.

Roberts and co-workers<sup>8</sup> found a close correlation between vitamin C intake and capillary fragility. They concluded that the capillary fragility test might be useful in showing relative saturation or unsaturation in given individuals or groups. Wright and Ludden<sup>9</sup> observed that subjects receiving inadequate amounts of vitamin C demonstrated increased capillary fragility before the classical signs of scurvy became manifest. Abt and associates<sup>10</sup>

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denied the existence of a significant correlation between plasma vitamin C level and petechial formation. It is now generally recognized that several clinical conditions other than scurvy (infectious disorders, acidosis, menstruation, blood dyscrasias, hypertension) as well as some toxic agents (arsphenamine, carbon monoxide) affect the strength of capillaries.

The fasting plasma vitamin C level has been widely accepted as a reliable biochemical index of the state of ascorbic acid nutrition. Farmer,<sup>11</sup> using the results of Thysell's<sup>12</sup> investigations, compiled a table which demonstrated the relationship between the dietary intake of vitamin C and the fasting plasma vitamin C level. Croft and Snorff<sup>13</sup> studied a group of fifty healthy student nurses on a regular hospital diet. The fasting plasma vitamin C values varied from 0.60 to 1.12 mg. per cent with an average of 0.81 mg. per cent. These figures compared favorably with those of Farmer and Abt<sup>14</sup> and of Portnoy and Wilkinson.<sup>2</sup> Kruse<sup>15</sup> maintained that no correlation existed between the data derived by different methods of vitamin C assay in the same patient. He stated that the practice of testing the validity of a procedure by comparing its results with blood values was unsound, since blood values shift rapidly and fluctuate intermittently while tissue concentrations undergo slow change.

The vitamin C saturation test has been found to be an excellent index of ascorbic acid deficiency. Wright and co-workers<sup>9, 16, 17</sup> and other investigators<sup>18-20</sup> have reported extensively on the various methods and procedures of vitamin C excretion tests. The former group derived a formula in which the one and one-half hour and five-hour urinary excretion of vitamin C after the intravenous administration of 1 Gm. was used to calculate the twenty-four hour saturation index. This index was shown to be more significant than the five-hour excretion value because the index nullified the factor of renal retention.

The present study was undertaken to correlate several of the more commonly employed procedures used to denote vitamin C unsaturation. This investigation embraces observations on the intradermal dye discoloration test, the capillary fragility test, the fasting plasma vitamin C level, and the vitamin C saturation test.

#### MATERIAL AND METHODS

Thirty-four patients admitted to the general medical service and fourteen normal volunteers (interns, nurses, and technicians) comprised the subjects used in this study. In each case the tests were run consecutively in the morning after an overnight fast. The evening meal on the day preceding the studies did not include foods rich in vitamin C. The order of testing was as follows: intradermal dye discoloration, capillary fragility, plasma vitamin C level, and the vitamin C saturation test.

The intradermal dye discoloration test was carried out simultaneously on both arms according to the procedure of Slobody.<sup>5</sup> The average of the two tests was taken as the dye discoloration time. The N/300 solution of 2,6 dichlorophenol-indophenol was not prepared freshly every day but was checked by titration against vitamin C for standardization twice weekly. The standards outlined by Slobody were employed. Accordingly, a dye discoloration time of more than fourteen minutes denoted a definite degree of vitamin C unsaturation, from nine to thirteen minutes mild unsaturation, and less than nine minutes a normal concentration of vitamin C in the tissues.

The capillary fragility test was performed and interpreted according to the procedure and standards outlined by Wright and Lilienfeld.<sup>21</sup> Less than ten petechiae, in a circle 2.5 cm. in diameter approximately 1 inch below the crease of the elbow after fifteen minutes of tourniquet pressure halfway between systolic and diastolic blood pressure, are considered normal; ten to twenty petechiae were interpreted as border line, whereas over twenty petechiae denoted definite evidence of increased capillary fragility.

The plasma vitamin C was determined by the Farmer and Abt metaphosphoric acid method.<sup>14</sup> The normal fasting level was considered to range from 0.6 to 1.4 mg. per 100 c.c. of plasma.

The vitamin C saturation studies\* were carried out according to the procedure of Wright and Ludden.<sup>9</sup> One gram of vitamin C was injected intravenously after a twelve-hour fast. Urine specimens were collected at one and one-half and five hours after the injection; the total five-hour excretion and the twenty-four hour saturation index were calculated. An excretion rate of over 450 mg. of vitamin C was considered normal for the five-hour period and over 500 mg. for the twenty-four hour saturation index.

Although Kruse<sup>15</sup> maintained that no correlation could be expected to exist between the various laboratory procedures for determining vitamin C nutritional status, it was felt, nevertheless, that some biochemical or biophysical test which most closely approximates the clinical status must be accepted as a standard in order to compare the relative adequacy of other procedures. This is particularly true if any one method is to be used exclusively in order to determine vitamin saturation or unsaturation. In this study the five-hour excretion test was adopted empirically as the method of choice, and the other indices compared with it. The twenty-four hour saturation index was not used as a basic standard since in several instances it was impossible to calculate this value. Moreover, in only one instance (Case 17) was there any discrepancy between the five-hour excretion value and the twenty-four hour saturation index.

## RESULTS

In Table I the observations made on the forty-eight cases studied are given in detail. This table was constructed according to the values obtained in the five-hour urinary excretion of vitamin C in ascending order.

This protocol reveals a lack of correlation between the intradermal dye discoloration time and the five-hour excretion of vitamin C in thirteen of the forty-eight individual tests performed (27 per cent). Only obvious discrepancies were used in these calculations since the borderline zone of nine to fourteen minutes in dye discoloration time was considered compatible with vitamin C saturation or unsaturation. The notable exceptions occurred in Cases 20 to 25, 29, 32, 34, 37, 38, 44, and 46.

Assuming the fasting vitamin C content of the plasma in nutritionally normal individuals to be at least 0.6 mg. per 100 c.c., a fair degree of correlation was noted between this index and the five-hour urinary excretion value. Obvious discrepancies were observed in seven of the forty-eight tests performed (15 per cent). The exceptions noted were in Cases 14, 17, 20, 22, 27, 34, and 37.

A marked degree of discrepancy was noted between capillary fragility and the five-hour urinary excretion of vitamin C. Here again, the borderline zone of ten to twenty petechiae was considered compatible with vitamin C saturation or unsaturation. Nevertheless, a lack of correlation between these two indices was observed in twenty-four of the forty-eight tests performed

\*With the technical assistance of Elsie V. Frost, B.A., and Margaret E. Thomas, B.A., of the Division of Pathological Chemistry, New York Post-Graduate Hospital.

TABLE I. VITAMIN C STUDIES IN FORTY-EIGHT SUBJECTS

CASE NO.	AGE	SEX	CLINICAL DIAGNOSIS	VITAMIN C SATURATION TEST		DYE DIS-COLORATION TIME (MIN. AND SEC.)	FASTING PLASMA VITAMIN C (MG. %)	CAPILLARY FRAGILITY (NUMBER OF PETECHIAE)
				FIVE-HOUR EXCRETION (MG. %)	TWENTY-FOUR HOUR SATURATION INDEX (MG. %)			
1	45	M	Chronic glomerulonephritis	118	189	17:30	0.15	100+
2	30	M	Peptic ulcer	154	161	22:45	0.10	0
3	21	F	Tuberculous peritonitis	156	203	19:00	0.25	8
4	23	M	Postphlebotic ulcer	180	186	19:30	0.10	0
5	44	M	Psychoneurosis	201	218	11:00	0.10	12
6	15	M	Pyrexia—etiology?	226	239	12:00	0.45	8
7	50	M	Chronic leg ulcer, rheumatoid arthritis	243	253	29:30	0.20	0
8	33	M	Chronic prostatitis	246	278	27:00	0.20	2
9	15	M	Possible early bronchiectasis	261	295	20:00	0.15	50
10	16	F	Obesity—etiology?	268	284	16:15	0.10	23
11	63	M	Thrombophlebitis	272	292	17:00	0.25	0
12	24	F	Multiple sclerosis	279		15:00	0.35	58
13	23	F	Brucellosis	325	352	14:30	0.20	80
14	26	M	Normal	349	397	15:00	0.70	3
15	60	F	Bleeding peptic ulcer	364		10:30	0.10	8
16	51	F	Hypertension, ovarian tumor	387	449	14:00	0.10	100+
17	58	F	Hypertension, hypertrophic osteoarthritis	412	538	10:00	0.75	40
18	56	F	Metastatic carcinoma with jaundice	417	468	13:00	0.25	48
19	20	F	Infectious arthritis	439	494	19:45	0.20	12
20	23	F	Postpolio lymphedema	487	545	21:00	0.40	1
21	16	F	Rheumatic heart disease	490		26:30	0.65	12
22	22	F	Normal	508	558	18:15	0.40	13
23	41	M	Rheumatic heart disease	510	679	19:15	0.65	48
24	27	M	Normal	556	603	21:00	0.80	20
25	26	F	Rheumatic heart disease (inactive), subacute bacterial endocarditis	556	739	16:45	0.75	1
26	39	M	Retinal hemorrhages—etiology?	566	692	10:15	0.80	100+
27	49	M	Coronary occlusion	592	760	14:00	0.35	19
28	56	M	Hypertension	620	736	6:30	0.70	90
29	24	F	Normal	624	754	14:30	1.00	5
30	40	F	Allergic to ragweed and citrus fruits	629	762	12:00	0.65	10
31	54	F	Hypertension, purpura	633	838	8:45	1.00	100+
32	50	F	Biliary cirrhosis	636	713	15:00	0.65	4
33	24	F	Rheumatic heart disease	647	829	11:30	0.80	4
34	21	F	Normal	658	792	29:30	0.55	25
35	47	F	Anemia—etiology?	668	873	8:30	0.95	15
36	40	F	Hyperthyroidism	679		11:00	0.70	5
37	22	F	Normal	686	804	15:15	0.40	56
38	27	M	Normal	701	941	18:15	0.90	4
39	52	F	Polycythemia vera	715	870	9:00	1.00	84
40	16	F	Multiple sclerosis	730	847	10:45	0.60	40
41	29	F	Normal	798	920	9:30	0.90	7
42	26	F	Normal	833	950	12:45	1.10	52
43	23	F	Normal	837	973	8:30	0.70	40
44	22	F	Normal	838	977	20:30	0.75	63
45	33	F	Thrombophlebitis	841	1,066	8:30	1.35	56
46	30	F	Normal	918	1,041	14:30	1.10	60
47	27	M	Normal	963	1,000	10:00	0.60	57
48	26	M	Normal	1,038		10:00	1.50	50



(50 per cent). The exceptions noted were in Cases 2 to 4, 6 to 8, 11, 14, 15, 23, 26, 28, 31, 34, 37, 39, 40, and 42 to 48. It should be pointed out here that in six patients (Cases 23, 26, 28, 31, 39, and 45) the discrepancy between capillary fragility and the five-hour saturation test may be ascribed to the underlying pathologic disturbance resulting in capillary injury (rheumatic heart disease, purpura, hypertension, polycythemia vera, and thrombophlebitis). In the eighteen remaining instances in which a lack of correlation was observed between these two procedures, no obvious reason to account for the discrepancy was disclosed. In the final analysis, therefore, no correlation was observed between the capillary fragility test and the five-hour saturation test in eighteen of the forty-eight tests performed (37 per cent).

#### DISCUSSION

The dye discoloration test exhibited serious technical difficulties and inconsistencies aside and apart from the high degree of discrepancy when compared with the saturation test. Thus, allergic reactions, such as induration and erythema at the site of the injection, were occasionally noted; these occurred twenty-four to ninety-six hours after the administration of the dye. In one subject the local reaction was marked and accompanied by a dermatitis involving the forehead, bridge of the nose, and flexor creases of the fingers. Moreover, significant variations in dye discoloration time were observed when the test was performed simultaneously on both arms. Peculiarly enough, this phenomenon could be repeated with identical results twenty-four hours later. Again, some difficulty was experienced in reading the absolute end point of dye discoloration, and some degree of uncertainty was always present concerning the validity of an absolute intradermal injection.

The capillary fragility test was found to be nonspecific. The present meager knowledge of capillary physiology and pathology renders any procedure based on capillary rupture extremely difficult to interpret. Massive petechial formation may occur in several clinical conditions (such as, blood dyscrasias) as well as in frank scurvy. Again, fragile capillaries produced by vitamin C deficiency will not rupture under tourniquet pressure if anemia is concomitantly present presumably due to the decreased blood volume.

#### CONCLUSIONS

1. The vitamin C saturation test, the intradermal dye discoloration time, the fasting plasma vitamin C level, and the capillary fragility test were carried out on thirty-four patients and fourteen normal subjects.

2. Serious discrepancies between the dye discoloration test and the five-hour urinary excretion of vitamin C were observed in 27 per cent of the tests performed. Other noteworthy objections to the intradermal dye discoloration test have been described.

3. A satisfactory correlation was observed between the fasting plasma level and the five-hour urinary excretion of vitamin C. In only 15 per cent of the cases studied were exceptions noted.

4. Obvious discrepancies between the capillary fragility and the saturation tests occurred in 37 per cent of the subjects investigated.

## REFERENCES

1. Rotter, H. (Budapest): Bestimmung des Vitamins C im lebenden Organismus, Wien. klin. Wehnschr. 51: 205, 1938.
2. Portnoy, B., and Wilkinson, J. F.: Intradermal Test for Vitamin C Deficiency, Brit. M. J. 1: 328, 1938.
3. Suzuki, S. (Mukden): Vitamin C and Skin Diseases; Intradermal Test With 2,6-dichlorophenolindophenol, J. Orient. Med. (Abstr. Sec.) 30: 267, 1939.
4. Beck, H., and Krieger, F. H.: Was leistet eine Intrakutanprobe mit Indophenol zur Prüfung des Vitamin C-Haushaltes? Deutsche med. Wehnschr. 65: 1336, 1939.
5. Slobody, L. B.: Intradermal Test for Vitamin C Subnutrition, J. LAB. & CLIN. MED. 29: 464, 1944.
6. Goldsmith, G. P., Gowe, D. F., and Ogaard, A. T.: Determination of Vitamin C Nutrition by Means of a Skin Test; a Critical Evaluation, Proc. Soc. Exper. Biol. & Med. 41: 370, 1939.
7. Wright, I. S., and MacLenathen, E.: The Intradermal Test for Vitamin C Determination, J. LAB. & CLIN. MED. 24: 806, 1939.
8. Roberts, L. J., Blair, R., and Bailey, M.: Seasonal Variations in Capillary Resistance of Institution Children, J. Pediat. 11: 626, 1937.
9. Wright, I. S., and Ludden, J. B.: Treatment With Vitamin C (Cevitamic Acid—Ascorbic Acid); Method for Compensating for the Factor of Error Due to Renal Retention of Vitamin C Found in All Previous Saturation and Blood Tests, M. Clin. North America 24: 743, 1940.
10. Abt, A. F., Farmer, C. J., and Epstein, I. M.: Normal Cevitamic (Ascorbic) Acid Determinations in Blood Plasma and Their Relationship to Capillary Resistance, J. Pediat. 8: 1, 1936.
11. Farmer, C. J.: Vitamin C Analysis in Relation to Clinical Problems, Quart. Bull., Northwestern Univ. M. School 14: 220, 1940.
12. Thysell, T.: C-Vitaminstandard und C-Hypovitaminose; über Kost, Blutascorbinsäure und Kapillarblutungsprobe, Acta paediat. 26: 481, 1939.
13. Croft, J. D., and Snorf, L. D.: Cevitamic Acid Deficiency; Frequency in a Group of 100 Unselected Patients, Am. J. M. Sc. 198: 403, 1939.
14. Farmer, C. J., and Abt, A. F.: Determination of Reduced Ascorbic Acid in Small Amounts of Blood, Proc. Soc. Exper. Biol. & Med. 34: 146, 1936.
15. Kruse, H. D.: A Concept of the Deficiency States, Milbank Mem. Fund Quart. 20: 245, 1942.
16. Wright, I. S., Lilienfeld, A., and MacLenathen, E.: Determination of Vitamin C Saturation; a 5 Hour Test After an Intravenous Test Dose, Arch. Int. Med. 60: 264, 1937.
17. Ludden, J. B., and Wright, I. S.: Effect of Renal Retention of Vitamin C on Saturation Tests; a Formula for Compensation of This Factor of Error, Arch. Int. Med. 65: 151, 1940.
18. van Eekelen, M.: On the Amount of Ascorbic Acid in Blood and Urine; the Daily Human Requirements for Ascorbic Acid, Biochem. J. 30: 2291, 1936.
19. Faulkner, J. M., and Taylor, F. H. L.: Observations on the Renal Threshold for Ascorbic Acid in Man, J. Clin. Investigation 17: 69, 1938.
20. Ralli, E. P., Friedman, G. J., and Rubin, S. H.: The Mechanism of the Excretion of Vitamin C by the Human Kidney, J. Clin. Investigation 17: 765, 1938.
21. Wright, I. S., and Lilienfeld, A.: Pharmacologic and Therapeutic Properties of Crystalline Vitamin C (Cevitamic Acid); With Especial Reference to Its Effects on the Capillary Fragility, Arch. Int. Med. 57: 241, 1936.

## SERUM PROTEINS IN HYPOPROTEINEMIA DUE TO NUTRITIONAL DEFICIENCY

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THE ultimate source of all body proteins is dietary protein. Dietary protein is absorbed from the gut as amino acids, and these acids are resynthesized into the special types of body protein. Inadequate intake of protein leads to a depletion of tissue protein. A sufficiently severe depletion of tissue protein is reflected in the serum proteins because there exists, between the cellular and serum proteins, a dynamic equilibrium involving a continuous interchange of materials between the blood and the tissues. Although the serum protein level is not an absolute indication of the state of the body proteins, hypoproteinemia reflects depletion of protein in other tissues.

Blood serum contains a variety of proteins which can be classified as albumin and globulins according to physical and chemical characteristics. Each performs distinct bodily functions in addition to the all-important but little-understood relationship to tissue nutrition. Serum albumin influences the water balance of the body. The edema which is often observed in cases of severe hypoproteinemia is thought to be due to a depletion of albumin below the level necessary for maintaining adequate colloid osmotic pressure within the vascular system.

Most studies on the problem of serum protein production have been confined to the synthesis of serum albumin because a reduction in the concentration of albumin usually precedes that of the globulins. The globulins are of vital importance because of their immunologic functions. Their complexity has been shown clearly by the fundamental work of Tiselius on the separation of plasma proteins by electrophoretic analysis. By this means the globulins have been divided into alpha, beta, and gamma components, and their quantitative relationship has been determined.

On the basis of electrophoretic analysis of blood serum of patients with a great variety of diseases, certain generalizations have been made. In febrile conditions the alpha globulins are elevated. In nephrosis the beta globulins are high, possibly because of the presence of a labile lipoprotein.<sup>1</sup> Marked increases in the gamma component occur in rheumatic fever,<sup>2</sup> amyloidosis,<sup>2</sup>

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relapsing malaria,<sup>3</sup> and sarcoidosis.<sup>4</sup> In certain diseases affecting the liver there is often an elevation of all three globulins.<sup>5</sup> Changes in the relative distribution of these proteins during the course of many diseases bear no direct relationship to total protein level, although the total protein level is not necessarily normal.

Although hypoproteinemia may result from severe hemorrhage, burns, or disturbances of digestion, absorption, and utilization of proteins, the most common cause of hypoproteinemia is insufficient intake of dietary protein. In a large Nutrition Clinic where hundreds of patients with various forms of malnutrition are seen yearly, many patients have been observed who, for years, have existed on a low protein diet and who have serum protein level 30 to 40 per cent below the normal value. The clinical implications of this type of hypoproteinemia are not well understood. In some of the advanced cases edema may occur, suggesting a depletion of serum albumin; but other cases show no signs of edema.

Although undernutrition and malnutrition have been recognized as contributing factors in chronic infectious diseases and in susceptibility to infection, observations of certain malnourished, anemic individuals at the Nutrition Clinic have revealed such patients to be remarkably free from infection despite low serum protein levels. In order to secure further data on these cases, electrophoretic patterns of blood serum, complement and complement components, opsonic indices, and natural antibodies have been compared with those of healthy subjects and with a series of patients with various other diseases.

#### MATERIALS AND METHODS

Three groups of subjects were selected for study. The first group included seven adults who had no evidence of any disease or infection. They appeared to be in excellent health; their diets were adequate in all respects, and they were working every day. The second group included fourteen malnourished patients, thirteen adults and one child selected from the Nutrition Clinic and from the wards of the Jefferson Hospital. These patients had various diseases (Table I), and it was known that their diets had been inadequate in protein for at least a short time. To what extent the infection was responsible for the poor nutritional status is not known. The third group included eight adults with anemia who had been observed in the Nutrition Clinic for at least three years. Three had addisonian pernicious anemia, one had nutritional macrocytic anemia, and four had iron deficiency anemia. All had subsisted on low protein diets for several years but were free from infection of any kind.

Fasting (overnight) samples of blood were drawn by venous puncture. The blood was allowed to clot, was centrifuged, and the serum was transferred to ampules in an ether-dry ice bath. Serum samples were preserved in the frozen state.

Electrophoretic analysis of all sera was carried out in a standard Tiselius apparatus according to Longsworth's technique.<sup>6</sup> A veronal buffer, pH 8.6, ionic strength 0.1 was employed. The serum was dialyzed forty-eight hours in 2

TABLE I. SERUM PROTEIN DISTRIBUTION ACCORDING TO TISELIUS DATA

CASE	SUBJECT	TOTAL PROTEIN (MG. %)	ALBUMIN (%)	ALPHA GLOBULIN (%)	BETA GLOBULIN (%)	GAMMA GLOBULIN (%)	A/G	REMARKS
<i>Normal Subjects</i>								
1	R. B. J.	6.60	62.1	9.6	11.5	16.9	1.6	Normal, healthy adults who showed no signs of infection at the time the blood sample was taken
2	M. B.	7.50	54.0	17.9	15.6	12.5	1.1	
3	S. D.	6.57	68.7	10.3	10.9	10.2	2.2	
4	A. M.	7.40	53.3	13.5	17.7	15.5	1.1	
5	H. C.	6.41	56.2	19.3	12.2	14.7	1.3	
6	L. F.	7.03	57.6	12.2	14.7	15.8	1.4	
7	D. J.	6.13	64.6	13.6	11.1	10.7	1.9	
Average		6.81	59.5	13.8	13.4	13.4	1.5	
Range		6.1 to 7.5	53 to 69	9 to 19	11 to 18	10 to 17	1.1 to 2.2	
<i>Subjects With Various Diseases</i>								
8	J. T.	6.13	43.3	20.8	21.4	14.4	0.76	Pulmonary edema; coronary ischemia
9	R. J.	5.88	45.6	13.5	18.2	22.6	0.84	Tuberculous meningitis
10	R. N.	6.71	23.7	29.1	19.0	28.1	0.31	Aleuemic leucemia
11	M. I. P.	7.94	22.5	19.7	6.8	51.0	0.29	Lymphopathia venereum
12	M. P.	4.94	62.3	12.0	16.1	9.6	1.6	Ulcers (staphylococcus infection)
13	J. W.	5.87	48.2	21.9	16.0	14.0	0.93	Catarrhal jaundice
14	W. R.	5.84	36.7	19.1	13.4	30.8	0.58	Typhus convalescent
15	F. C.	5.09	47.5	21.2	15.0	16.2	0.91	Active tuberculosis
16	I. B.	6.25	33.0	26.7	17.4	22.9	0.49	Lung abscess
17	J. T. S.	7.72	33.0	29.0	16.5	21.5	0.49	Catarrhal jaundice
18	J. K.	6.25	28.1	27.4	21.0	23.5	0.39	Rheumatic fever
19	F. B.	4.06	30.0	30.6	20.7	18.6	0.43	Nephritis; congestive heart failure
20	M. R.	5.87	23.2	11.8	13.7	51.1	0.35	Carcinomatous pellagrous dermatitis
21	R. G.	3.09	43.6	30.5	20.9	5.0	0.77	Hepatitis (?)
Range		3.1 to 7.9	23 to 62	12 to 31	6.8 to 21	5 to 51	0.29 to 1.6	
<i>Subjects With Anemia</i>								
22	E. S.	5.10	57.4	12.5	11.9	18.0	1.4	Nutritional macrocytic anemia
23	G. B.	4.71	60.8	10.0	12.3	16.6	1.5	Addisonian pernicious anemia
24	J. S.	4.59	63.8	7.3	11.2	17.6	1.8	Addisonian pernicious anemia
25	H. T.	5.75	53.6	13.6	13.3	19.5	1.2	Addisonian pernicious anemia
26	C. D.	4.12	53.1	18.1	15.2	13.6	1.1	Iron deficiency anemia
27	H. G.	5.75	54.6	13.2	20.7	11.6	1.2	Iron deficiency anemia
28	E. L.	5.04	56.2	14.3	15.3	14.3	1.3	Iron deficiency anemia
29	S. H.	5.85	50.9	13.0	17.8	20.3	1.0	Iron deficiency anemia
Average		5.11	56.3	12.8	14.7	16.4	1.3	
Range		4.1 to 5.8	51 to 64	7 to 18	11 to 21	13 to 20	1.0 to 1.8	

liters of buffer; then the serum protein concentration was adjusted to 1.2 per cent by dilution with buffer. Electrophoresis was allowed to proceed for 9,300 seconds with a current of 15 milliamperes. Results were recorded photographically by the *Schlieren* scanning method.<sup>7</sup> Measurements were made in terms of relative quantities (weight per cent) of albumin and alpha, beta, and gamma globulins by means of planimeter tracings of enlargements of the photographic plates. Descending patterns only were used for the calculations.

Total proteins were determined by the micro-Kjeldahl method for nitrogen. A sulfuric acid-phosphoric acid-copper sulfate digestion mixture was used, and Nessler's reagent was employed for the colorimetric estimation of the ammonia

formed. Tungstate sulfate precipitation was used in the preparation of protein-free filtrates for N.P.N. determinations.

For a qualitative study of the serum proteins, the immunologic properties of each serum were measured with respect to three well-known natural attributes of serum: complementary activity, opsonic index, and antishoop antibody titer.

The components of hemolytic complement have been shown to be distributed among the globulin fractions of the serum proteins.<sup>8</sup> Although the complementary activity of the blood determines to a large extent its immunologic properties, a deficit of any or all of the components of complement causes no obvious changes in the electrophoretic pattern of the serum since complement comprises only a small portion of the serum globulins. For this reason complement titers are included in our data. Complement determinations were carried out as soon as possible after the serum had been prepared, since complementary activity decreases gradually even when the serum is preserved in the frozen state. The serial tube method described by Ecker and associates<sup>9</sup> was used. The values represent the amount of serum necessary to bring about complete hemolysis and 50 per cent hemolysis of a standard amount of sensitized sheep red blood cells. Tests for the reactivation of components were carried out for all sera. These values are reported in terms of per cent hemolysis of a given amount of sheep cells brought about by small amounts of serum to which an excess of specifically inactivated complement components were added.

The opsonic index of serum was measured by the degree of opsonization of organisms which do not undergo phagocytosis in the absence of an opsonic agent. In the absence of antibodies natural opsonins have been identified with three of the components of complement.<sup>10</sup> They represent an important mechanism in reaction to infectious agents; therefore, measurement of opsonic indices is helpful in the evaluation of the serum globulins. Determinations were made according to the method described by Ecker and Lopez-Castro.<sup>10</sup> All leucocytes were obtained from the venous blood of the same normal individual. The organism used was *Micrococcus candidus*.<sup>\*</sup> The number of cocci ingested by 100 polymorphonuclear leucocytes was counted on each slide.

In addition to the antibodies which are formed in response to invasion, human blood serum normally contains agglutinins which are generally active in low dilutions against certain cellular antigens. The antigen used in this study was the sheep erythrocyte. The sera to be tested were heated at 56° C. for ten minutes to inactivate complement. Serial tube dilutions from 1:2 to 1:128 were then made with 0.85 per cent saline in such a manner that the total volume in each tube was 0.5 cubic centimeter. A 2 per cent suspension of sheep erythrocytes was prepared from 2 cubic centimeters fresh sheep blood washed four times with saline and diluted to 100 cubic centimeters. To each tube of diluted serum and to a "blank" tube containing 0.5 cubic centimeter saline was added 0.5 cubic centimeter of this cell suspension. The tubes were shaken, incubated one

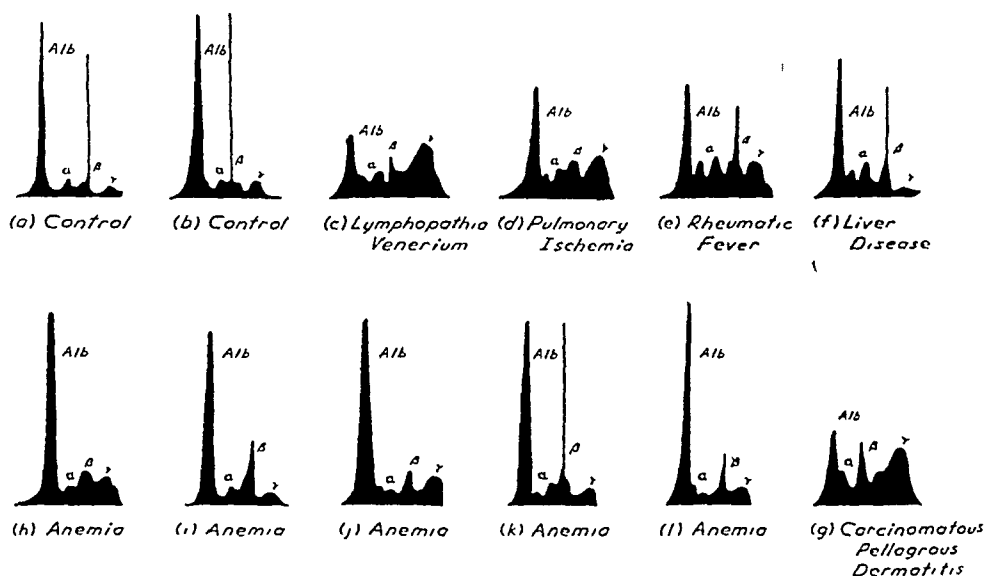
<sup>\*</sup>*Micrococcus candidus* was obtained through the kindness of Dr. C. A. Mills, University of Cincinnati Medical School.

hour at 37° C., and allowed to stand overnight in an icebox. Readings were made according to the size of the erythrocyte clump remaining after the tube had been tapped firmly once. The values reported represent the highest dilution of serum capable of causing definite agglutination of the sheep cells.

# DATA AND RESULTS

*Serum Protein Fractionation by Electrophoresis.*—Electrophoretic data are presented in Table I. Typical patterns are illustrated in Fig. 1. The average values for normal controls are in agreement with the data published by other

## TYPICAL ELECTROPHORETIC PATTERNS



A1b = Albumin ;  $\alpha$ ,  $\beta$ , and  $\gamma$  refer to those globulin fractions

Fig. 1.—Note similarity of the patterns for the controls a and b and the anemia patients h, i, j, and k, as compared with the differences between the patterns for the controls and the patterns for patients with various diseases c, d, e, f, and g.

workers<sup>11</sup> as are the data for the patients with various other diseases.<sup>2, 3, 5</sup> The patterns for the patients suffering from anemia and hypoproteinemia resemble very closely the normal patterns. The only consistent variation is a slight elevation of the gamma globulins with a relative drop in either albumin or alpha globulin.

*Immunologic Tests.*—Contained in Table II are the results of complement titrations and reactivations, natural antish sheep antibody determinations, and opsonic indices.

Values for 50 per cent hemolysis readings in the complement titrations are within the normal range for all cases with the exception of No. 8. Reactivation tests on this serum indicate that there was a relative deficit of all complement

CASE	SUBJECT	COMPLEMENT													OPSONIC INDEX	NATURAL ANTIBODIES
		TITER		REACTIVATION												
		50% HEMOL. YSIS	100% HEMOL. YSIS	0.02 C.O. SERUM	+SUPER	+PPT	+Z	+NH <sub>4</sub>	0.10 C.O. SERUM	+SUPER	+PPT	+Z	+NH <sub>4</sub>			
1	R. B. J.	.05	.22	0	50	0	10	30	Tr	95	30	30	85	954/96	250	
2	M. B.	.03	.07	Tr	50	25	10	60	30	90	60	50	80	1570/100	16	
3	S. D.	.04	.18	Tr	85	25	10	50	15	95	60	50	90	795/95	16	
4	A. M.	.03	.08	Tr	30	20	10	50	10	90	50	35	90	541/90	16	
5	H. C.	.04	.16	0	55	15	Tr	60	10	95	30	50	90	1406/94	64	
6	L. F.	.05	.18	0	45	20	15	50	15	90	40	45	85	1291/99	64	
7	D. J.	.04	.18	0	40	10	30	40	20	60	30	50	50	642/93	4	
Range		.03 to .05	.07 to .22	0 to Tr	30 to 850	0 to 25	Tr to 30	30 to 60	Tr to 35	60 to 95	30 to 60	30 to 50	50 to 90	541 to 1570	4 to 256	
Normal Subjects																
Subjects With Various Diseases																
8	J. T.	Inc		0	30	0	30	Tr	0	55	Tr	5	35	2304/90	4	
9	R. J.	.12	.30	0	40	10	25	25	10	60	40	35	50	825/95	16	
10	R. N.	.03	.16	0	70	10	10	35	30	100	60	50	90	1259/97	8	
11	M. I. P.	.02	.06	Tr	55	25	10	55	15	95	60	50	90	1354/98	64	
12	M. P.	.03	.10	Tr	55	Tr	30	30	20	85	60	50	70	1265/97	64	
13	J. N.	.04	.18	Tr	40	Tr	25	Tr	10	80	15	40	30	1751/100	8	
14	W. R.	.05	.14	Tr	50	Tr	30	35	15	70	40	50	70	—	16	
15	F. C.	.04	.12	0	60	5	10	20	40	60	20	40	70	1157/99	—	
16	I. B.	.04	.10	0	50	5	10	10	10	65	10	20	40	1542/100	64	
17	J. T. S.	.05	.33	0	50	5	10	10	10	25	75	40	70	1070/95	32	
18	J. K.	.04	.14	0	50	20	30	40	25	75	40	50	70	—	32	
19	F. B.	.06	.18	0	50	Tr	5	15	5	60	10	20	50	—	32	
20	M. R.	.05	.14		75	25	30	50	25	90	65	75	80	—	128	
21	R. G.	.05	.18	5	40 to 75	Tr to 25	5 to 30	Tr to 55	0 to 40	55 to 100	Tr to 60	5 to 50	30 to 90	825 to 2304	8 to 128	
Range		.06 to .12	.12 to .33	0 to 5	40 to 75	Tr to 25	5 to 30	Tr to 55	0 to 40	55 to 100	Tr to 60	5 to 50	30 to 90	825 to 2304	8 to 128	
Subjects With Anemia																
22	E. S.	.05	.12	0	60	15	10	20	Tr	90	30	20	65	—	8	
23	G. B.	.05	.12	0	60	15	10	30	Tr	95	30	20	75	936/91	4	
24	J. S.	.05	.14	0	60	10	10	20	Tr	95	30	20	70	528/80	16	
25	H. T.	.04	Inc	0	60	0	20	20	15	95	30	40	60	1156/98	64	
26	C. D.	.02	.12	5	60	20	15	65	20	95	70	95	95	1297/96	64	
27	H. G.	.03	.36	0	50	0	20	30	25	90	30	50	55	1344/99	8	
28	E. L.	.03	.10	0	60	10	10	30	Tr	95	30	40	80	52/19	2	
29	S. H.	.08	.21	0	50	10	30	45	30	55	25	50	60	910/91	32	
Range		.02 to .08	.10 to .36	0 to 5	50 to 60	0 to 20	10 to 30	20 to 65	Tr to 30	55 to 95	25 to 70	20 to 90	55 to 95	52 to 1344	2 to 64	

Complement titers, expressed as cubic centimeter serum required to bring about 50 per cent and 100 per cent hemolysis under the condition of the titration.

Reactivation, expressed as per cent hemolysis.

Super, the supernatant after treatment of normal serum with a phosphate buffer, pH 6.1, ionic strength 0.02. This fraction lacks the C'1 component of complement.

Ppt, the precipitate after treatment of normal serum with phosphate buffer. This fraction lacks the C'2 component of complement.

Z, the fraction of normal serum remaining after treatment with zymosan. This fraction lacks the C'3 component of complement.

Opsonic index, expressed as number of organisms engulfed by 100 P.M.N./number of leucocytes containing organisms.

Natural antibodies, expressed as the highest dilution of serum containing sufficient antibodies to cause visible agglutination of sheep cells.

Tr, trace (very slight hemolysis).

Inc, incomplete (incomplete hemolysis).



components. Reactivation tests on the other sera showed no significant variations in strength of components, as was to be expected, since over-all complement titers were within the normal range.

The antish sheep hemagglutinin titers were somewhat lower for the deficient patients than for either group of controls. However, the ranges of values overlapped widely, and small numerical variations are within the range of experimental error.

In most cases the opsonic index counts showed 90 to 100 per cent of the polymorphonuclear leucocytes to be filled with organisms and the total number of organisms engulfed to be 500 to 1,500. In Case 28 this value was substantially lowered, indicating the absence of adequate quantities of opsonins in this serum. "Blank" controls showed not more than 10 per cent active leucocytes and not more than twenty-five organisms engulfed.

#### COMMENT

The data presented indicate that the blood sera of eight persons who are hypoproteinemic but have no known infection vary but slightly from the sera of healthy, well-nourished individuals with respect to distribution of serum protein fractions, complement titers, opsonic indices, and antish sheep hemagglutinin titers.

The relative decrease of serum albumin and relative elevation of serum globulin fractions in the sera of most of the hypoproteinemic patients are an indication that hypoproteinemia develops at greater expense to serum albumin than to serum globulin. However, the average deviation of the albumin:globulin ratio from the normal is extremely small as compared with the wide deviations and inversions of the ratio found in the sera of patients with other diseases. This fact suggests that in prolonged malnutrition a gradual depletion of all serum proteins occurs, since the ultimate source of all body proteins is dietary protein. Whereas, in disease increased amounts of specialized globulins may be produced, in dietary deficiency all types of serum protein may be produced in normal quantitative proportions.

Although complement titer is known to undergo extreme variations in the course of certain infectious diseases<sup>12</sup> the titers for all but one case studied here remained normal. Case 8 (J. T.) was the only patient who was acutely ill at the time when the blood sample was taken. Apparently the components of complement are maintained at a normal level even when other protein levels decrease. Similarly the normal opsonic index values found for deficient patients suggests such a condition, since three components of complement (C'1, C'2, C'4) together may operate as an opsonin. The variation of the natural antish sheep antibody titers between the controls and the deficient patients is of questionable significance. Thus, the results of three immunologic reactions show that the sera of persons who are hypoproteinemic but have no infection can maintain certain immunologic functions even though a rather large proportion of the total serum protein has disappeared.

Reports concerning the relationship between nutritional status and resistance to infection are at great variance. In the case of hypoproteinemia due to acute starvation, hemorrhage, or surgical operation, resistance to infection is greatly lowered, and direct loss of antibody protein is probably the contributing factor. Apparently malnutrition presents a different problem, for many malnourished patients with anemia who appear to be as resistant to certain diseases as well-nourished individuals have been observed in the Nutrition Clinic. A partial explanation has been found in the increased phagocytic activity of the neutrophils in patients with anemia.<sup>13</sup> A complement to this explanation may be found in the results of the present study; that is, that in persons where hypoproteinemia exists because of a lack of materials with which to manufacture serum proteins, the limited amount of materials available contributes to the production of certain important immunologic elements.

#### SUMMARY AND CONCLUSIONS

1. Various characteristics of the serum proteins of twenty-nine individuals have been studied. Seven were from apparently normal, healthy adults; fourteen were from hospital patients with various diseases; and eight were from patients with hypoproteinemia associated with nutritional anemia but showing no evidence of infection.

2. Electrophoretic analysis demonstrated that the sera of the healthy individuals compare closely with the normal values recorded by other investigators. The sera of the patients with various diseases showed the abnormalities usually observed in those diseases, while the sera of the patients with anemia and hypoproteinemia differed from the normal only by a slight relative decrease in the amount of albumin present.

3. Complement titers, opsonic indices, and antishoop hemagglutinin titers of all sera showed no significant variation from the established normal values.

4. The gradual depletion of serum protein levels which follows chronic, uncomplicated malnutrition is probably a relative depletion of all types of protein distinguishable by electrophoretic analysis. Certain portions of the globulins appear to be maintained. This fact may afford a partial explanation of the resistance to infection shown by some persons who are hypoproteinemic because of dietary deficiency.

#### REFERENCES

1. Longsworth, L. G., and MacInnes, D. A.: An Electrophoretic Study of Nephrotic Sera and Urine, *J. Exper. Med.* 71: 77, 1940.
2. Leutscher, J. A.: Electrophoretic Analysis of Plasma and Urinary Proteins, *J. Clin. Investigation* 19: 313, 1940.
3. Fisher, A. M., and Davis, B. D.: The Serum Proteins in Sarcoid, *Bull. Johns Hopkins Hosp.* 71: 364, 1942.
4. Dole, V. P., and Emerson, K.: Electrophoretic Changes in Plasma Protein Patterns in Patients With Relapsing Malaria, *J. Clin. Investigation* 24: 644, 1945.
5. Gray, S. J., Barron, E. S. C.: The Electrophoretic Analysis of Serum Proteins in Diseases of the Liver, *J. Clin. Investigation* 22: 191, 1943.
6. Longsworth, L. G., and MacInnes, D. A.: Electrophoresis of Proteins by the Tiselius Method, *Chem. Rev.* 24: 271, 1939.

7. Longsworth, L. G., Shedlovsky, T., and MacInnes, D. A.: Electrophoretic Patterns of Normal and Pathological Human Blood Serum and Plasma, *J. Exper. Med.* 70: 399, 1939.
8. Pillemer, L., Ecker, E. E., Oncley, J. L., and Cohn, E. J.: The Preparation and Physicochemical Characterization of the Serum Protein Components of Complement, *J. Exper. Med.* 74: 297, 1941.
9. Ecker, E. E., Pillemer, L., and Seifter, S.: Immunochemical Studies on Human Serum. I. Human Complement and Its Components, *J. Immunol.* 47: 181, 1943.
10. Ecker, E. E., and Lopez-Castro, G.: Unpublished data.
11. Moore, D. H., and Lynn, John: Electrophoretic Measurements on Normal Human Plasma, *J. Biol. Chem.* 141: 819, 1941.
12. Ecker, E. E., Seifter, S., Dozois, T. F., and Barr, L.: Complement in Infectious Diseases. Unpublished.
13. Berry, L. J., Davis, J., and Spies, T. D.: Phagocytic Activity of Neutrophils in Anemias, *J. LAB. & CLIN. MED.* 30: 910, 1945.

# FOLIC ACID IN THE TREATMENT OF APLASTIC ANEMIA

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MANY authors<sup>1, 2</sup> have shown the value of folic acid in the treatment of pernicious anemia and a variety of other macrocytic anemias. However, reports on the use of folic acid in the treatment of aplastic, hypoplastic, or refractory anemias have shown disappointing results. Watson and co-workers<sup>3</sup> reported their results with folic acid in the treatment of refractory anemia. They treated seven patients using 2.5 mg. twice daily for six days, and at the end of that time no beneficial effect was observed. Spies<sup>4</sup> found folic acid administration to be without effect on either the clinical or hematologic picture of three patients with aplastic anemia. Doan<sup>4</sup> also reported negative results in six patients with hypoplastic anemia. The dosage used and the duration of treatment were not described. Zuelzer,<sup>5</sup> discussing the beneficial effect of folic acid in macrocytic anemia of infancy, mentioned negative results obtained in three children with aplastic or hypoplastic anemia. Goldsmith<sup>6</sup> has recently reported on the treatment with folic acid of two patients with aplastic anemia. One patient, a 51-year-old colored man, received 5 mg. and later 30 mg. of *Lactobacillus casei* factor daily. At the end of one month there had been no improvement and it was necessary to administer several blood transfusions. The second patient, an 8-year-old white boy, received 20 mg. of *L. casei* factor parenterally for ten days and then 40 mg. daily for a similar period. There was no change in the blood picture. On the other hand, in 1944 Sharp and associates<sup>7</sup> reported on the treatment of ten patients with refractory anemia who were given small daily doses of Vitamin B<sub>12</sub> (0.6 to 1.5 mg.). After a month there was an appreciable increase in the hematocrit but otherwise only slight changes, and the experiment was discontinued. Recently Davidson and Girdwood<sup>8</sup> have reported some beneficial effect following folic acid administration in the treatment of three patients with refractory megaloblastic anemia in whom adequate therapy with parenteral concentrated liver was ineffectual. Since folic acid has not seemed to have any therapeutic value in the treatment of aplastic anemia according to the experience of most authors, it would seem of value to record our experiences with the use of folic acid in which the apparent results of treatment have seemed a little more hopeful.

In the summer of 1945 we were faced with the problem of a patient severely ill with aplastic anemia who was having frequent transfusion reactions. This patient had already received over seventy blood transfusions in the preceding eight months and required about 500 c.c. of whole blood weekly in order to maintain a red count of 2.5 million. There had been a history of sulfonamide treatment; therefore, at the suggestion of Dr. Louis K. Diamond of Boston, we decided to observe the effects of folic acid in the treatment of this condition

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Later, when more folic acid was made available, two other patients were treated; this report summarizes the effect of this treatment and discusses the pertinent experimental data.

### CASE REPORTS

**CASE 1.**—A 29-year-old white man, a lieutenant in the Air Corps, was well until December, 1944, when he developed a sore throat and fever while on shipboard en route to the United States. He was admitted to the sick bay, and a right tonsillar ulcer was found with regional cervical lymph node enlargement. A smear was reported positive for Vincent's infection, and he was treated with sulfadiazine orally, a single 0.6 Gm. injection of neoarsphenamine intravenously, and neoarsphenamine in glycerin topically. The patient received a total of 7 Gm. of sulfadiazine by Dec. 4, 1944, when, because of continued illness, a blood count was taken; the count revealed: hemoglobin, 7.6 Gm.; red blood cells, 2,700,000 per cubic millimeter; white blood cells, 2,150 per cubic millimeter; neutrophils, 19 per cent. Medication was discontinued. On Dec. 7, 1944, a sternal marrow aspiration was done and revealed an aplasia of all cellular elements. Petechiae developed. Treatment consisted of repeated blood transfusions, penicillin, and pentnucleotide injections with some improvement. The patient received sixteen transfusions until his admission to Lovell General Hospital on Dec. 20, 1944. On admission the patient was still severely ill and was complaining of sore throat, weakness, and rectal pain.

**Past History.**—There was a history of atabrine ingestion of 0.1 Gm. daily for several months after April, 1944. Sulfadiazine had been administered on many previous occasions for repeated upper respiratory infections, and in addition, sulfadiazine ointment had been used freely for various cuts and abrasions during the preceding year.

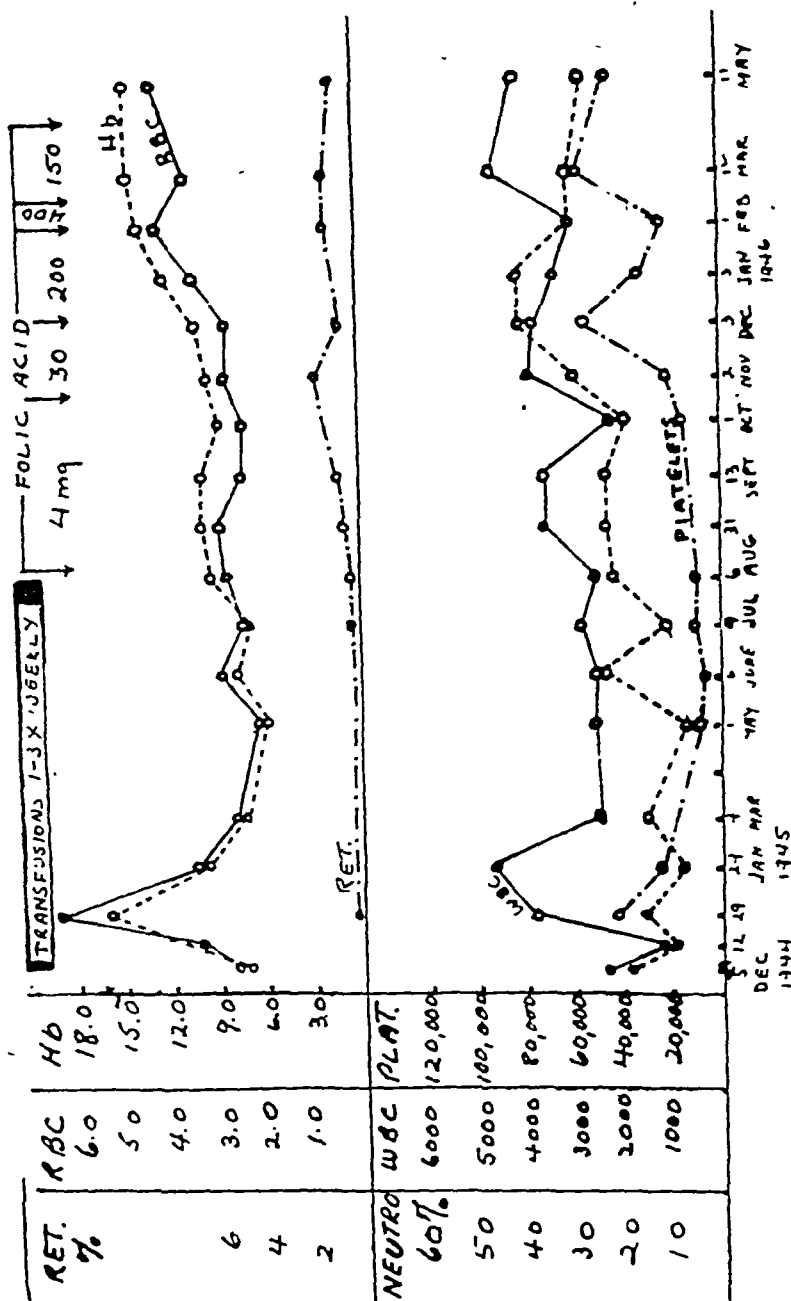
**Physical Examination.**—A moderate pallor and a periumbilical scaly eruption were present. There were no enlarged lymph nodes and the liver and spleen were not palpable. Multiple anal fissures were present. The remainder of the examination was not significant. No petechiae or ecchymoses were noted on admission.

**Laboratory Data.**—In Fig. 1 are summarized the blood counts taken during the patient's hospital stay. The initial marked increase in red cell count and hemoglobin found at this hospital was attributed to the large number of transfusions given in the short time prior to admission. Sternal puncture on Dec. 29, 1944, revealed a hypoplastic marrow with a depression of the erythroid and myeloid elements and an absence of megakaryocytes.

**Course.**—The patient was severely ill during most of the hospital stay which lasted fifteen months. He developed petechiae, ecchymoses, epistaxis, and a hematoma of the eyelid on various occasions. In January, 1945, ulceration of the mucous membranes of the mouth was noted. In May, 1945, the patient developed a hemorrhage into the right middle ear. During hospitalization he received penicillin for intercurrent infections and over seventy blood transfusions, from one to three weekly until August, 1945. During July and August, 1945, transfusions were accompanied by a febrile and constitutional reaction but no hemolytic reaction. Careful examination for abnormal agglutinins was done and none were found. (This examination was performed by Dr. L. K. Diamond at the Blood Grouping Laboratory, Boston, Mass.) Because of the frequency of transfusion reactions and the progressive fall in the blood without them, it was decided to institute therapy with folic acid\* and to try to discontinue transfusions. In August, 1945, all transfusions were stopped and none were given subsequently. Administration of folic acid, 4 mg. daily, was started. There was no reticulocytosis and no improvement in the blood picture, but the count did not drop and folic acid was continued. On Oct. 18, 1945, the dosage of folic acid was increased to 30 mg. daily and on December 6, 1945, to 200 mg. daily. During this time the red count slowly improved, although the leucocyte and platelet counts were essentially unchanged. In February, 1946, the dosage was increased to 400 mg. daily for two weeks and then was reduced to 150 mg.

\*Folic acid used in all three cases in the present study was synthetic *L. casei* factor (pteroylglutamic acid) and was given by mouth.

daily. Folic acid was discontinued in April, 1946. In May, 1946, a blood count revealed: hemoglobin, 13.8 Gm.; red cells, 4,000,000; white cells, 4,000; neutrophils, 28 per cent; platelets, 54,000. The patient felt well and was ambulatory.



*Past History.*—The patient received four sulfadiazine tablets on two or three occasions in August, 1944, because of an epidemic of meningitis in the camp at which he was stationed. Again, prior to going overseas in December, 1944, he was given several tablets of sulfadiazine for an unknown reason. There was no other history of exposure to toxic chemicals.

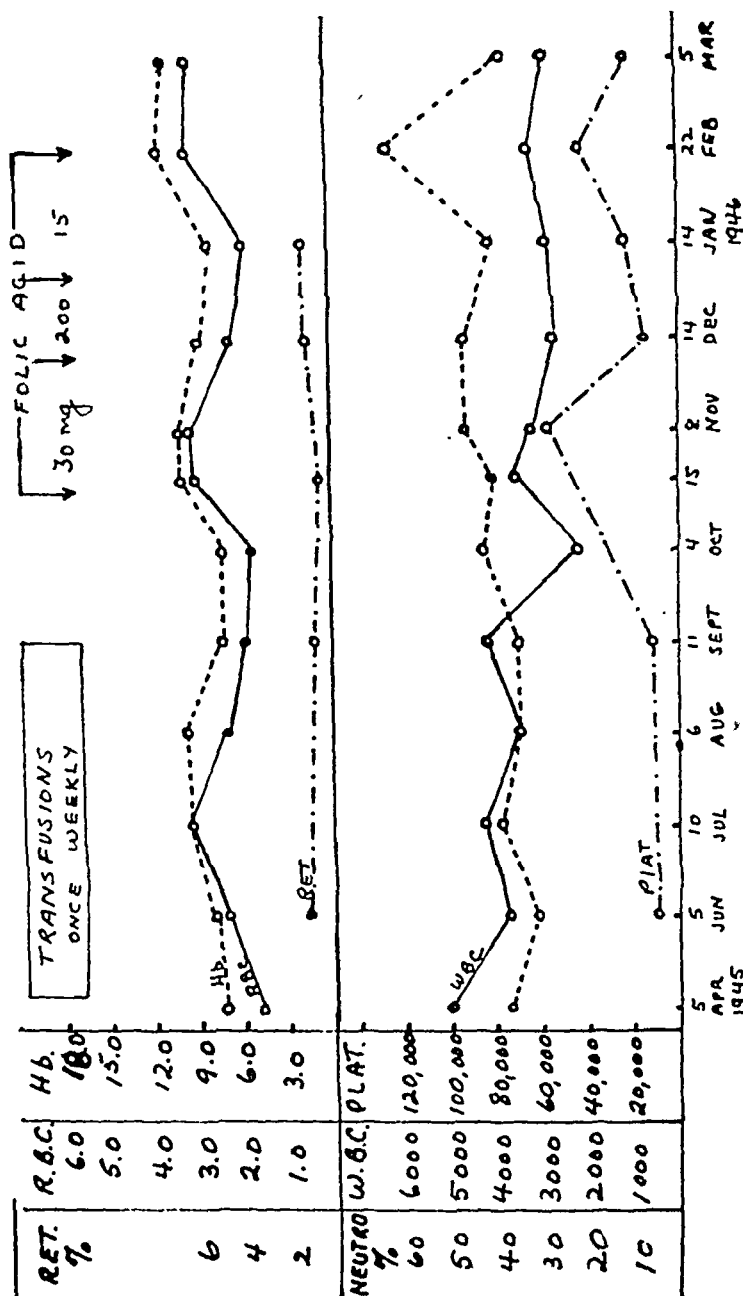


Fig. 2.—Summary of hematologic data in Case 2.

*Physical Examination.*—Hemorrhages were noted in the fundi, more marked in the left eye. A moderate pallor was present. There was no lymph node enlargement and liver and spleen were not palpable. There were no other significant physical findings.

*Laboratory Data.*—In Fig. 2 are summarized the blood counts taken during the patient's hospital stay. In June, 1945, a sternal marrow aspiration revealed a hyperplasia of the normoblastic and myeloid elements and a normal number of megakaryocytes. Gastric analysis revealed 42 units of free HCl after the administration of histamine.

*Course.*—The patient was given four blood transfusions and evacuated to the United States. Subsequently, he received transfusions about once a week from June until September, 1945. In early September, 1945, he developed abdominal pain, slight fever of 100 to 101° F., tenderness of the liver, and an icterus index of 20. There was no increase in anemia nor any other evidences of a hemolytic crisis. The condition persisted for ten days and returned to normal. It was believed that the patient suffered a mild episode of homologous serum jaundice, induced by a previous transfusion. On Oct. 13, 1945, transfusions were discontinued and the patient was started on 30 mg. of folic acid per day. This was continued until Dec. 6, 1945, when the dosage was increased to 200 mg. daily. On Jan. 1, 1946, the dosage was decreased to a maintenance dose of 15 mg. daily which was continued until February, 1946. On this regime the blood count fluctuated at about the previous levels, although the patient did not receive any of the blood transfusions which were previously necessary to maintain the blood count. No data are available concerning the further course.

CASE 3.—A 25-year-old white man, a technician fifth grade, was well until the middle of June, 1945. At that time he noted the gradual onset of fatigue. Shortly thereafter he developed shortness of breath and anorexia. On July 15, 1945, he fainted and consequently was hospitalized.

*Past History.*—The patient served overseas in the Southwest Pacific Theater of Operations for twenty-six months. The diet was considered adequate, and there were no gastrointestinal complaints until the patient developed anorexia two weeks prior to hospitalization. Atabrine, 0.1 Gm. five times weekly, was administered during the entire stay overseas.

*Physical Examination.*—A marked pallor was noted. There was a symmetrical pinkish scaling involving the chest, abdomen, and neck. Bluish bands were noted under the finger- and toenails. There was also a bluish discoloration of the skin under the eyes and over the right shin. A slight generalized lymph node enlargement was noted and liver and spleen were not palpable. The remainder of the examination was noncontributory.

*Laboratory Data.*—In Fig. 3 are summarized the blood counts taken during the patient's hospital stay. Fragility test was normal, icterus index was 5, and urinary urobilinogen was positive in 1:20. Stools were negative for occult blood. Gastric analysis revealed the presence of free HCl. Kahn was negative. A trephine biopsy of the sternum revealed a hypoplastic marrow.

*Course.*—The patient received fourteen blood transfusions of 500 c.c. each during July and August, 1945. This produced an improvement in the general condition and blood picture. The leucocytes and platelets became essentially normal, but the red cell count was fixed between 2.5 and 3.5 million. This persisted from September until December, 1945, without significant change, despite the administration of iron and parenteral liver. On Dec. 6, 1945, the administration of 200 mg. of folic acid (50 mg. four times daily) was begun. This was continued until Dec. 21, 1945, when the drug was discontinued. On this regime a progressive rise in the red count to normal was noted without any significant increase in reticulocyte count. After discharge from the hospital, a follow-up letter was received from the patient stating that he was enjoying good health and that the blood count on July 3, 1946, was normal.

#### DISCUSSION

The condition presented by these three patients was classified as aplastic anemia. Two of our patients (Cases 1 and 3) had a bone marrow which revealed a hypoplasia of the blood-forming elements. This, together with the peripheral blood picture of pancytopenia, would satisfy even the most rigid diagnostic criteria for this disorder. However, our second patient (Case 2) differed in that the marrow was slightly hyperplastic. The precise classification of this



type of anemia is obscure, although many authorities include them with the aplastic anemias.<sup>9, 10</sup> It is for this reason, and as a matter of convenience, that our three cases are considered together.

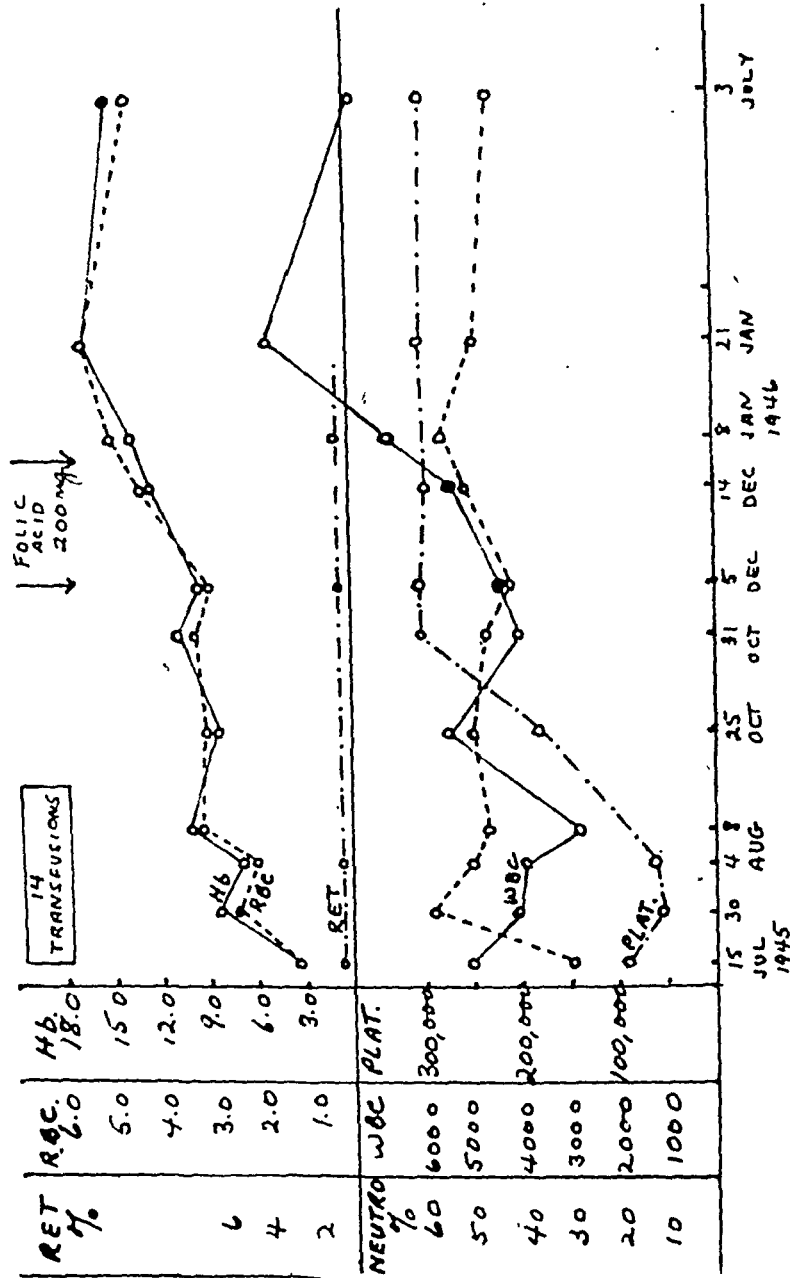


Fig. 3.—Summary of hematologic data in Case 3.

It was extremely difficult to evaluate the etiologic factors in the pathogenesis of the aplastic anemia in these patients. In our first patient there was a history of ingestion of a number of different medications. Atabrine was

taken for suppression of malaria during the summer months. However, it is unlikely that it was related to the development of the aplastic anemia, because such cases were not seen in patients from the Mediterranean Theater of Operations where atabrine was taken only for a few months during the year. There was also a history of the administration of 7.0 Gm. of sulfadiazine in about two days. On numerous previous occasions our first patient had taken sulfadiazine for a variety of infections and had abundant opportunity to develop a drug sensitivity. In addition, he also received an intravenous injection of 0.6 Gm. of neosarsphenamine and neosarsphenamine in glycerin was applied topically to the tonsillar ulcer. Whether or not these drugs given singly or combined were responsible for this condition cannot be stated with any degree of certainty. There is a strong possibility that the patient may have had an aplastic anemia from the onset and that the tonsillar ulcer was a manifestation of this disease. Unfortunately, no blood counts were obtained until after the drugs were administered, when it was obvious that the patient was not improving.

The second patient also had a history of sulfadiazine ingestion, but the latent period was too long before the onset of symptoms for one to consider it seriously as a causative factor. The third patient gave a history of prolonged ingestion of atabrine while overseas in the Southwest Pacific Theater of Operations. He also showed the lesions of atypical lichen planus which are currently believed to result from atabrine toxicity.<sup>11</sup> Some patients with atypical lichen planus have also developed visceral damage including aplastic anemia.<sup>11, 12</sup> It is possible but not certain that atabrine played some role in the development of the condition of our third patient.

The rationale for the trial of folic acid in the treatment of aplastic anemia was based on the experimental production of anemia, leucopenia, and granulocytopenia in rats on sulfonamide therapy. This peripheral blood picture was similar to the pancytopenia of aplastic anemia. In addition, it has been shown that folic acid deficiency in rats was accompanied by a progressive hypoplasia of the marrow with a depletion of all the marrow elements.<sup>13</sup> In extreme cases aplasia of the marrow was produced. Thus, it is seen that, experimentally in rats, deficiency of folic acid produces an aplastic anemia.

The results obtained in these three patients were not as striking as the improvement shown by folic acid-deficient rats in which there was a return to a normal blood picture after four days of treatment. In macrocytic anemias the results are also more striking, and a reticulocyte response may be demonstrated similar to the one obtained from adequate liver therapy. No significant reticulocytosis was manifest in any of these patients. In our first patient we were having difficulty with transfusions which he required at least once a week in order to maintain a satisfactory blood count. He showed a gradual improvement in the red count, which rose to better levels than we could maintain on transfusions. Improvement was such that he was transformed from a bedridden patient to one who was ambulatory and capable of ordinary activity. However, a complete remission had not been attained, as evidenced chiefly by the continued low platelet count and also by the persistent leucopenia and

granulocytopenia. In our second patient we were able to dispense with transfusions, although the count did not rise to better levels. The third patient already had had a partial remission, as evidenced by a normal sternal marrow aspiration, normal white count and differential, and normal platelet count prior to starting therapy with folic acid. The red count remained low after several months of observation and did not rise after attempts at therapy with iron and liver extract. Following the administration of folic acid, the patient had a remission which he has maintained for eight months after the completion of treatment.

It is realized that the improvement of these three patients may very well be a coincidental spontaneous remission, and there is evidence in our third patient that a remission had started before folic acid was administered. However, it is possible that folic acid may have had some beneficial effects on the course of the disease, and it is believed that a trial of folic acid using large doses over a long period of time is justified.

#### CONCLUSIONS

1. Three patients with aplastic anemia were treated with folic acid.
2. Treatment was associated with a remission of varying degree in each instance.
3. It is recommended that large doses used over a long period of time be given a trial in the treatment of this condition.

#### REFERENCES

1. Spies, T. D.: Effect of Folic Acid on Persons With Macrocytic Anemia in Relapse, *J. A. M. A.* 130: 474, 1946.
2. Moore, C. V., Bierbaum, O. S., Welch, A. D., and Wright, L. D.: The Activity of Synthetic Lactobacillus Casei Factor ("Folic Acid") as an Antipernicious Anemia Substance. I. Observations on Four Patients: Two With Addisonian Pernicious Anemia, One With Nontropical Sprue, and One With Pernicious Anemia of Pregnancy, *J. LAB. & CLIN. MED.* 30: 1056, 1945.
3. Watson, C. J., Sebrell, W. H., McKelvey, J. L., and Daft, F. S.: Possible Effectiveness of L. Casei Factor ("Folic Acid") Concentrates on Refractory Anemia and Leukopenia, With Particular Reference to Leukopenia Following Radiation Therapy, *Am. J. M. Sc.* 210: 463, 1945.
4. Doan, C. A.: Folic Acid (Synthetic L. Casei Factor), an Essential Panhematopoietic Stimulus. Experimental and Clinical Studies, *Am. J. M. Sc.* 212: 257, 1946.
5. Zuelzer, W. W.: Folic Acid Therapy in the Anemias of Infancy and Childhood, *J. A. M. A.* 131: 137, 1946.
6. Goldsmith, G.: The Treatment of Macrocytic Anemia With Lactobacillus Casei Factor (Pteroylglutamic Acid), *J. LAB. & CLIN. MED.* 31: 1186, 1946.
7. Sharp, E. A., Vonder Heide, E. C., and Wolters, J. G.: Preliminary Clinical Observations on the Anti-Anemia Vitamin B<sub>12</sub> (Yeast Concentrate), *J. A. M. A.* 124: 734, 1944.
8. Davidson, L. S. P., and Girdwood, R. H.: Folic Acid in the Treatment of Megaloblastic Anemia, *Lancet* 251: 373, 1946.
9. Wintrobe, M. M.: *Clinical Hematology*, Philadelphia, 1942, Lea & Febiger.
10. Kracke, R. R.: *Diseases of the Blood and Atlas of Hematology*, ed. 2, Philadelphia, 1941, J. B. Lippincott Company.
11. Special Article: Evaluation of the Untoward Reactions Attributable to Atabrine, *Bull. U. S. Army M. Dept.* 4: 653, 1945.
12. Personal observation.
13. Endicott, K. M., Daft, F. S., and Ott, M.: The Bone Marrow in "Folic Acid" Deficiency and Its Response to Crystalline Lactobacillus Casei Factor ("Folic Acid"), *Arch. Path.* 40: 364, 1945.

## APLASTIC ANEMIA FOLLOWING ADMINISTRATION OF THOROTRAST

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SINCE thorium dioxide has come to be used extensively as a diagnostic test for visualization of the liver, spleen, placenta, ureter, kidney pelvis, and certain blood vessels,<sup>21</sup> it is important to record in the literature any toxic reactions which seem to have occurred as a result of the thorium administration. We have recently had an opportunity to study a patient with hypoplastic anemia who had been given thorium dioxide in usual diagnostic amounts nine years previous to the development of the hypoplastic anemia; there were presumptive reasons for thinking that the thorium might possibly have caused the hypoplasia.

Reports have appeared in the literature which indicate that thorium dioxide (thorotrast) might have late toxic manifestations of a serious nature. This toxicity may present itself with irritative or destructive effects, primarily on reticulo-endothelial and hematopoietic tissues; however, other tissues are involved, such as the kidney, where a nephritis has been produced experimentally.<sup>12, 15</sup> Martland and associates,<sup>2, 3, 10</sup> Gettler and Norris,<sup>4</sup> Sabin and co-workers,<sup>11</sup> and Foulds<sup>14</sup> have observed the development of osteogenic sarcoma clinically and experimentally from the use of radioactive materials, principally radium, although thorium is seen to produce similar effects. Efsskind<sup>15</sup> produced papillomatous growths by the intraperitoneal injection of thorium. These same investigators, in addition to Lambin and Gérard,<sup>7</sup> Gottlieb,<sup>6</sup> and Meakins,<sup>5</sup> have demonstrated aplastic anemia or hypoplastic anemia with destruction and hypoplasia of hematopoietic tissue following thorium administration. The sarcomatous changes usually develop later than the aplastic anemia, the sarcoma developing between ten to twenty years and the aplastic anemia developing five to ten years after the administration of the radioactive material.<sup>9</sup>

Martland and associates<sup>2, 3, 10</sup> further state that thorium is a more active disrupter of blood centers than radium. Investigators are cognizant that a patient may remain in good health for many years before toxic manifestations appear. Descriptions of such cases in the literature, however, are not numerous, and not all observers are in agreement that the amounts of thorium used clinically may be injurious.<sup>6, 8</sup> The case presented here serves further to illustrate the evolution of an aplastic anemia following the use of radioactive thorium.

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## CASE REPORT

A 53-year-old colored woman was admitted to the hospital Oct. 7, 1944, with a chief complaint of fever and persistent headache.

One week before entry the patient noted onset of a persistent headache. Accompanying the headache there developed fever and dizziness. Shortly before this there had been some anorexia and weight loss. The severity of these symptoms had increased progressively up to the time of admission. The family history was essentially negative. The patient had been in an automobile accident twenty-two years prior to admission, after which a left hemiplegia was present with left ear deafness and paralysis of cranial nerves VI and VII. She had typhoid fever in childhood and nine years before hospitalization was studied for abdominal pain, at which time liver visualization with thorotrast was done. We are unable to find from the record how much thorotrast was given, but there is no reason to suspect that it had been more than the standard dose. A diagnosis of peptic ulcer was eventually made and a gastroenterostomy was performed. The patient's discomfort then disappeared. The catamenia was negative.

In the isolation department on admission, a negative spinal fluid was obtained. On physical examination the temperature was 99.5° F., pulse 116 per minute, respiratory rate 25 per minute, and blood pressure 114/50. The patient appeared acutely ill. The mucous membranes were pale. Two large, firm nodules, 3 cm. in diameter, were found, one in each antecubital fossa. There was a left-sided hemiplegia. The fundus of each eye showed marked A-V nicking and silver wire arteries. A small area of ulceration was found in the right tonsillar fossa. The thyroid was somewhat enlarged and nodular, and the neck veins were distended. Dullness and increased breath sounds were found in the middle one-third of the left lung area. The heart was markedly enlarged, the apex being in the sixth interspace, 12 cm. from the midsternal line, and a loud, harsh systolic murmur was heard over the apex and entire precordium. The abdomen and the pelvis revealed no abnormal physical signs.

*Laboratory Data.*—

Blood findings on Oct. 8, 1944, were: hemoglobin, 3.0 Gm. per 100 c.c.; red blood cells, 1.26 million per cubic millimeter (hypochromic, poikilocytic, and anisocytic); white blood cells, 450 to 950; hematocrit cell volume, 8 per cent; differential—lymphocytes, 88 per cent, neutrophils, 2, bands, 1, juveniles, 8, basophiles, 1.

Blood findings on Oct. 10, 1944, were: red blood cells, 1.25 million; white blood cells, 210; hemoglobin, 4.1 grams.

Urinalysis on Oct. 9, 1944, revealed: specific gravity, 1.010; albumin, trace.

On Oct. 9, 1944, a sternal marrow study showed marked reduction in all nucleated elements as well as platelets. A blood culture was negative at this time.

On Oct. 10, 1944, an x-ray revealed a small contracted spleen and negative chest. On Oct. 11, 1944, blood cultures showed large gram-negative rods; additional cultures of blood revealed similar growths. On Oct. 12, 1944, a positive agglutination was obtained with Friedländer's bacillus Group A serum. The organism was noncapsulated. The patient died on this day.

There was nothing in the patient's history to indicate that she ever had been exposed to any hematopoietic toxin other than thorium dioxide.

*Necropsy.*—The body was that of a well-developed somewhat emaciated colored woman. A few pigmented areas were seen over the anterior aspect of both lower extremities. A well-healed linear scar was found in the upper right abdominal quadrant. There was no cyanosis, edema, jaundice, or lymph node enlargement. Pin-point petechiae were present over the chest. No free fluid was found in the peritoneal or pleural cavities. There were adhesions between the abdominal scar and the transverse mesocolon. The liver extended 3 cm. below the right costal margin and the spleen was very small. The apex of the left lung was bound by stringy fibrous adhesions to the parietal pleura. The heart weighed 320 grams and was flabby in consistency. There was no hypertrophy or dilatation of the chambers and the valves were negative. The coronary vessels were patent and showed only an occasional atheromatous plaque. The lungs together weighed 800 grams and were

edematous and congested, the left lower lobe showing some bronchopneumonia. The liver weighed 1,400 grams and was congested with slight peripheral "nutmegging." Fifteen to twenty pea-sized, dark green calculi were found in the gall bladder; these were soft and friable. The spleen weighed 15 grams, had a wrinkled thickened capsule, was rubbery in consistency, and on section showed a delicate grayish white dense framework. The pancreas and adrenals were negative. The kidneys together weighed 250 grams. Their capsules stripped easily and showed pale red surfaces. The corticomedullary ratios and demarcations were not disturbed. A slight white grayish streaking was present in the cortices. The pelvis showed pin-point hemorrhages. The uterus had a pedunculated fibromyoma, 2 cm. in diameter, and the left ovary contained a hazelnut-sized cyst. A few hemorrhagic areas were seen in the stomach and the large bowel on their mucosal surfaces. A rubbery blood clot, which was firmly attached to a hemorrhagic area, was found in the peripyloric region. The thyroid was enlarged and nodular. Nodules were seen which were encapsulated; some had undergone cystic degeneration. The bone showed osteoporotic trabeculae and pale gray somewhat moist marrow.

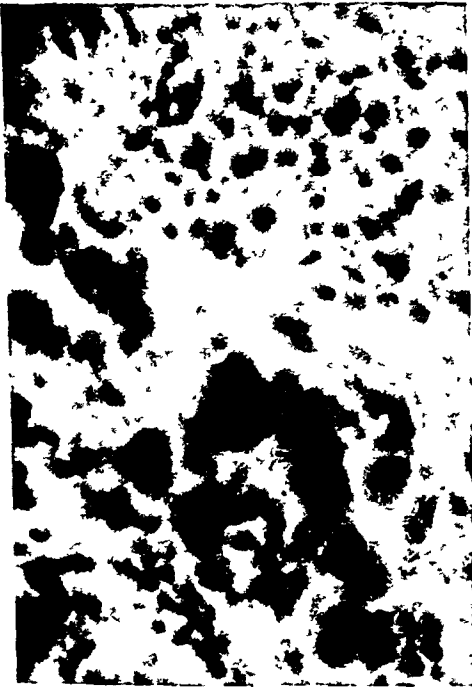


Fig 1—Thorotrast in splenic pulp (histiocytes).



Fig. 2.—Thorotrast in bone marrow cells.

*Histopathology.*—The section of uterus shows a typical fibromyoma. There is lipomatosis and congestion of the pancreas. Tubular degeneration and desquamation of the kidney with vasculization of the glomeruli and beginning nephrosclerosis is found. Scattered necrotic areas surrounded by lymphocytes are in the liver; there are however, no giant cells. Around these areas and about the central veins and portal fields is a grayish dark translucent pigment. Pigment is also seen in hepatic and Kupffer cells. There is widening of trabeculae in the spleen and marked fibrosis of the pulp. Large amounts of grayish dark translucent pigment are present in the pulp and capsule (Fig. 1).

The splenic capsule is thickened and fibrotic. The lung tissues show focal areas of emphysema and other areas of focal fibrosis and congestion. The adrenals are partly autolyzed. A mediastinal lymph node contains caseous necrosis and foci of fibrosis and

congestion. A grayish dark translucent pigment is seen here also which does not appear to be anthracotic. The ovary shows focal fibrosis. The heart shows myocardial fragmentation. The bone marrow is aplastic and contains dark gray translucent pigment (Fig. 2).

*Radioactivity.*—The radioactivity of samples of the bone marrow and spleen were determined by one of us (W.D.U.). This was accomplished with a special design of Geiger-Mueller counter in such a way as to afford comparisons with standard preparations of thorium by the alpha particle plus beta ray count and independently by the gamma ray count.

In addition to these two methods of making the comparison, the tissues were examined in two states. For the preliminary measurements the formalin was removed and the tissues were dried and powdered. For the final determinations the powdered tissues were ignited at 800° C., leaving only the inorganic oxides of the radioactive and other materials. In Table I under the fifth column is shown a good agreement between the content of thorium oxide determined on the dried tissue and on the ignited residues. In Table I also is demonstrated an abnormally high content of inorganic material (7.9 per cent by weight) in the bone marrow, not all of which is thorium oxide as shown in the sixth column of the table.

TABLE I. CONTENT OF THORIUM OXIDE BY WEIGHT IN TWO TISSUES FOLLOWING ADMINISTRATION OF THOROTRAST

TISSUE	STATE OF SAMPLE	PER CENT OF THORIUM OXIDE IN SAMPLE	PER CENT OF SAMPLE IN ORIGINAL TISSUE	PER CENT OF THORIUM OXIDE IN ORIGINAL TISSUE	PER CENT OF ALL OTHER INORGANIC OXIDES IN ORIGINAL TISSUE
Bone marrow	Dried	23.0	22.10	5.10	3.10
	Ignited	60.0	7.90	4.80	
Spleen	Dried	1.2	23.20	0.28	0.34
	Ignited	42.0	0.59	0.25	

Thorium, like uranium, is the progenitor of an established series of a number of radio elements. Radium is but one of these elements in the uranium series. The radioactivity of a sample of thorium arises largely from the disintegration of a succession of radio elements each derived in turn from its preceding parent. In old preparations, such as the standards used here, equilibrium will have been established and the average number of disintegrations in a given time will be the same for all the members. This is not true if the preparation has been disturbed chemically, but it is unfortunate that, from the viewpoint of the clinical use of thorotrast, the radioactivity of a preparation of thorium can never be reduced below about one-half of the maximum because an important member of the series (namely, radiothorium) is chemically identical with thorium itself (an isotope).

A comparison of the gamma ray measurements and the alpha particle plus beta ray determinations on the tissues in this case indicated that the thorium oxide had been undisturbed chemically for about nine years. This calculation was performed without knowledge of the case history, and it is a gratifying

TABLE II. DENSITY OF RADIATION IN TWO TISSUES EXPRESSED AS TOTAL EMISSION OF EACH TYPE OF RAY PER MINUTE PER GRAM OF TISSUE IN FORMALIN STATE

TYPE OF RADIATION	BONE MARROW	SPLEEN
Alpha particles	43,300	2,200
Beta rays	25,800	1,300
Gamma rays (primary)	28,000	1,400

confirmation of the measurements to note that administration of the thorotrast occurred 10.2 years before the date of the radioactive determinations.

The density of the radiation to which the tissue was being subjected was calculated for the three types of radiation from the data in Table I and the known radioactive constants for the elements in the thorium series, taking into account the state of radioactive equilibrium after nine years. The results are given in Table II.

Not all the rays of a given type in Table II possess equal energies. In studies of the pathologic effects of radiation a definite physical unit for the energy generated should be employed. Such a unit is the ergs per hour per gram of dry tissue. For the thorium series, after nine years, the energy per thorium alpha particle plus that of the alpha particles and beta rays from the decay products is  $44 \times 10^{-6}$  erg. The energy derived from the gamma rays is negligible and in any case is not dissipated locally as will be discussed. On this basis the thorium oxide in 1 Gm. of dried bone marrow produced 123 ergs per hour and 1 Gm. of dried spleen 6.3 ergs per hour. Some of this energy may be lost to the tissue by exhalation through the lungs of the sixth element in the series which is a gas (namely, thoron); however, according to measurements on the exhalation of radon,<sup>1</sup> an isotope of thoron, there could not be more than a 40 per cent reduction in the energy of radiation received by the tissue.

According to the literature<sup>10, 23</sup> numerous examples of acute symptoms have been observed, where the dosage of radiation energy in the body tissues, for comparable lengths of time, was even less than in the spleen in this case.

Radiation from thorium: Thorium dioxide, the agent used in this case for diagnostic purposes, is usually marketed as thorotrast, a colloidal sol of thorium dioxide (25 per cent). As far as radioactivity is concerned, there is no difference, except for the numerical values of the radioactive constants, between thorium and the members of its series and uranium and its members, one of which is radium. Both series emit all of the same three types of rays, alpha particles, beta rays, and gamma rays. The alpha particle is a positively charged nucleus of a helium atom and possesses comparatively great kinetic energy by virtue of its mass and high velocity of 12 to 18 thousand miles per second. However, this energy is completely dissipated in a very short distance, not more than a few tens of microns in tissue. Beta rays consist of electrons of much smaller mass but greater velocities approaching that of the speed of light. Their maximum energy is on the average about  $\frac{1}{10}$  of that of the alpha particles, but some travel up to  $\frac{1}{2}$  mm. in tissue before this energy is completely dissipated. Gamma rays are quanta of energy, similar in all respects to x-rays except for the much higher frequency of vibration. They travel with the speed of light and most of them possess sufficient energy to pass completely through the human body. The bio-



logic effects of alpha, beta, and gamma rays are said to be correlated in the following numerical values: 10,000, 100, and 1, respectively.

It is important to realize from what has just been said that there is a very great difference between the external and internal application of one and the same source of radiation in the type and degree of radiation to which the parts of the body may be subjected. The human body can be subjected without harm to surprisingly large quantities of radiating material placed in its immediate vicinity for short periods of time; time is an important element. The reasons for this are: (1) gamma rays are the only ones which effectively enter the body tissues on external radiation (except in the case of proximity to a cyclotron or fission process when a large and hazardous dose of neutrons may be experienced in addition to gamma radiation), and (2) the body receives only that part of the radiation within the solid angle subtended with the source. The same quantities administered internally have often proved fatal.

In internal administration of material such as thorium, whose decay products emit all three types of rays, the toxic effects within the body tissues are due almost entirely to alpha particles. The action of the gamma rays is negligible, and by comparison with the effects of the alpha particles, the action of the beta rays can be dismissed as minimal.

#### THE DISTRIBUTION AND ACTION OF ADMINISTERED THORIUM

The distribution of thorium is dependent upon the route of administration. Following intravenous injection it is first taken up by capillary endothelium and then phagocytized by the cells of the reticulo-endothelial and hematopoietic systems, including liver, spleen, bone marrow, lymph nodes, lungs, gonads, suprarenals, and placenta, but it is not transmitted to fetus.<sup>3, 8, 20-22</sup>

The excretion of the thorium is comparatively rapid from the liver and spleen; however, its diminution from bone marrow is extended over a long period of time, portions of the administered material remaining indefinitely in the marrow. Following disintegration of the thorium into its corresponding series of elements, thoron, a gaseous radio element, is eliminated by the lungs. Partial excretion of the thorium dioxide takes place through the kidneys.<sup>3, 8, 9, 16, 21</sup>

In the liver the Kupffer cells and hepatic cells are seen to hold quantities of thorium, while the monocytic cells and sinusoidal spaces of the spleen contain thorium after its administration.<sup>3, 8, 9, 16, 20-22</sup>

The irritative and destructive effects with concomitant fibroblastic proliferation and regenerative hyperplasia result in replacement of normal tissues, as well as causing cell maturation arrest and interference with nuclear mitosis. Subsequently abnormal cellular elements appear.<sup>3, 20</sup>

The grayish dark translucent pigment found in the bone marrow, liver, spleen, and lymph nodes corresponds to the usual classical pathologic description of thorotrast in the tissues. This histopathologic observation, as well as the studies of the radioactivity, demonstrates the presence of thorium in this case. The mechanism of the development of aplastic anemia is well confirmed

in the literature as arising from the radiation of hematopoietic tissues, and it may be presumed that such a mechanism was operative in this case.

The problem remains to establish an explanation for the fact that many patients suffer no harmful effects following the use of radioactive material. We cannot offer an explanation other than that which is discussed by others, namely, that there is an individual susceptibility to the radiation. However, we do propose that, besides individual susceptibility, there may be differences in the mode and speed of excretion in different persons which cause retention of more radioactive material in some than in others.

#### SUMMARY AND CONCLUSIONS

The existence of an aplastic anemia in our case is established beyond doubt. Measurements of radioactivity in the formalin-fixed tissues verify with equal certainty the presence of radioactive material in the body. In addition, histopathologic appearance of liver, spleen, and bone marrow sections reveals a grayish black diffusely distributed pigment within the sections, which according to present knowledge could be diagnosed only as being thorotrast. An attempt has been made to deduce that the radiations arising from the thorium series of radio elements could have been responsible for the aplastic anemia and fatality.

The literature concerning the toxicity of radioactive materials is reviewed so as to demonstrate the actual occurrence of untoward effects. Also, a brief résumé of the action of thorium in the body is undertaken in the hope that it may clarify the actual participation of radioactive agents in pathologic changes in the body tissues.

#### REFERENCES

1. Evans, R. D.: Radium Poisoning; Quantitative Determination of Radium Content and Radium Elimination Rate of Living Persons, *Am. J. Roentgenol.* 37: 368-378, 1937.
2. Martland, H. S., Conlon, P., and Knef, J. P.: Unrecognized Dangers in the Use and Handling of Radioactive Substances; With Especial Reference to Storage of Insoluble Products of Radium and Mesothorium in the Reticulo-Endothelial System, *J. A. M. A.* 85: 1769-1776, 1925.
3. Martland, H. S.: *Cecil—Textbook of Medicine*, ed. 6, Philadelphia and London, 1943, W. B. Saunders Company, pp. 523-527.
4. Gettler, A. O., and Norris, C.: Poisoning From Drinking Radium Water, *J. A. M. A.* 100: 400-402, 1933.
5. Meakins, J. C.: *The Practice of Medicine*, ed. 3, St. Louis, 1940, The C. V. Mosby Company, p. 713.
6. Gottlieb, R.: Effect of Colloidal Thorium on the Blood Picture, *Canad. M. A. J.* 28: 496-497, 1933.
7. Lambin, P., and Gérard, M. J.: La thrombopénie causée par le dioxyde de thorium colloidal, *Sang* 6: 804-808, 1932.
8. Harrison, B. J. M.: *A Textbook of Roentgenology: The Roentgen Ray in Diagnosis and Treatment*, Baltimore, 1936, William Wood & Company, p. 367.
9. Evans, R. D., Harris, R. S., and Bunker, J. W. M.: Radium Metabolism in Rats, and the Production of Osteogenic Sarcoma by Experimental Radium Poisoning, *Am. J. Roentgenol.* 52: 353-373, 1944.
10. Martland, H. S.: Occurrence of Malignancy in Radioactive Persons; General Review of Data Gathered in the Study of Radium Dial Painters, With Special Reference to Occurrence of Osteogenic Sarcoma and Interrelationship of Certain Blood Diseases, *Am. J. Cancer* 15: 2435-2516, 1931.
11. Sabin, F. R., Doan, C. A., and Forkner, C. E.: Production of Osteogenic Sarcomata and Effects on Lymph Nodes and Bone Marrow of Intravenous Injections of Radium Chloride and Mesothorium in Rabbits, *J. Exper. Med.* 56: 267-290, 1932.

12. Adams, L. J., Egloff, W. C., and O'Hare, J. P.: Experimental Chronic Nephritis Produced by Radium, *Arch. Path.* 15: 465-489, 1933.
13. Efskind, L.: Örtliche Veränderungen bei intraperitonealer Injektion von Thoriumdioxyd (Thorotrast), *Acta chir. Scandinav.* 84: 79-95, 1940.
14. Foulds, L.: Production of Transplantable Carcinoma and Sarcoma in Guinea-Pigs by Injections of Thorotrast, *Am. J. Cancer* 35: 363-373, 1939.
15. Fleming, A. J., and Chase, W. H.: Effects of Administration of Thorium Dioxide, *Surg., Gynec. & Obst.* 63: 145-148, 1936.
16. Jones, H. B., Wrobel, C. J., and Lyons, W. R.: Method of Distributing Beta-radiation to Reticulo-Endothelial System and Adjacent Tissues, *J. Clin. Investigation* 23: 783-788, 1944.
17. Gates, O.: Effects of Radiation on Normal Tissues; Effects on Bone, Cartilage and Teeth, *Arch. Path.* 35: 323-340, 1943.
18. Selling, L. S., and Osgood, E. E.: In Downey, H. (editor): *Handbook of Hematology*, Vol. 4, New York, 1938, Paul B. Hoeber, Inc., pp. 2693-2801.
19. Gould, B. S.: Effects of Thorium on Blood and Liver Enzymes of White Rats, *Proc. Soc. Exper. Biol. & Med.* 35: 77-78, 1936.
20. Wen, I. C., and Jung, T. S.: Reactions of Blood and Tissue Cells to Colloidal Thorium Dioxide, *Chinese J. Physiol.* 8: 85-94, 1934.
21. Best, C. H., and Taylor, N. B.: *The Physiological Basis of Medical Practice*, ed. 4, Baltimore, 1945, Williams & Wilkins Company, pp. 81-82.
22. Sollman, T.: *Manual of Pharmac.*, ed. 6, Philadelphia and London, 1939, W. B. Saunders Company, pp. 523-527.
23. Martland, H. S., and Humphries, R. E.: Osteogenic Sarcoma in Dial Painting Using Luminous Paint, *Arch. Path.* 7: 406-417, 1929.

## STUDIES ON LEUCOCYTE GRANULES AFTER STAINING WITH SUDAN BLACK B AND MAY-GRÜNWALD GIEMSA

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**I**N MANY types of clinical and investigative work, it frequently becomes necessary to locate lipids in tissues. Several types of dye can be used for this purpose but those of the sudan series, particularly sudan III and IV, have become especially popular. When frozen sections (or other materials which have not been subjected to the action of fat solvents) are stained with a solution of one of these dyes, the lipid inclusions are specifically reddened. The density and shade with which these inclusions are colored depend somewhat on the technique, the chemical nature of the lipid, and the particular lot of dye. About a decade ago Lison<sup>1</sup> discovered that still another member of this dye family, sudan black B, can be successfully employed as a histologic lipid stain. After treatment with a solution of this stain, lipid inclusions are specifically blackened.

In his original paper Lison<sup>1</sup> discusses in detail some of the technical problems associated with the use of red dyes. He notes that when inclusions are stained in red (that is, with sudan III or IV) there is no common cytoplasmic dye which brings them into satisfactory contrast. If the inclusions are large and/or numerous, lack of sharp contrast is not particularly detrimental; should they be small and few, however, this factor adds to the difficulty of locating and studying them under the microscope. Optical differentiation, the author points out, is greatly improved by use of sudan black B, for the blackened lipid masses are sharply contrasted by any of the common cytoplasmic stains.

Sudan black B has been used not only on tissue sections but also for staining of bacterial films (Hartman<sup>2</sup> and Burdon and associates<sup>3</sup>) and blood films (Sheehan<sup>4</sup>). We shall be particularly concerned with the latter type of preparation in the present investigations.

In his rather brief report Sheehan<sup>4</sup> has noted that definitive granules of both mature and immature leucocytes are blackened by exposure to sudan black B, while other parts of these cells are unaffected. With his technique, granules of eosinophile leucocytes, for example, appear to stain only on their surfaces, giving one the impression that they are hollow in nature. This author has found also that some myeloblasts contain sudanophilic granules while others are entirely sudanophobic in reaction.

The foregoing method for staining leucocyte granules is, however, of limited usefulness because of the difficulty of accurately identifying the cells concerned. This is especially true of immature leucocytes where finer details of cytoarchitecture and tinctorial reaction are prime criteria for differentiation of cell types.

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It is often found that the dye combinations commonly used for staining blood films are not entirely adequate for study and differentiation of various granular inclusions in leucocytes. For this reason an attempt was made to devise a combined technique, wherein a well-standardized method (May-Grünwald Giemsa) was used to bring out the main structural features of leucocytes and a relatively new dye (sudan black B) to stain their granular inclusions. It will be shown later that this combined technique retains most of the advantages of the May-Grünwald Giemsa method and at the same time brings out granule morphology especially well.

In testing this new method, paired films of normal and pathologic blood were made; one member of each pair was stained with May-Grünwald Giemsa and the other with the sudan May-Grünwald Giemsa combination. Comparisons then were made on leucocytes in the two types of preparation, and an attempt was made to determine the effects of the sudan stain. Later, some films were stained with sudan alone (as suggested by Sheehan) and used to check the findings. By these methods we were able to note that certain features of leucocyte morphology can be most profitably studied only after preparation of films by the combined technique.

#### MATERIALS AND METHODS

After a number of trials it was found that dried blood films can be advantageously studied after fixation in formalin-alcohol and staining with sudan black B and May-Grünwald Giemsa. These films were treated in the following manner:

1. Fix thoroughly dried (preferably for twenty-four hours) blood films for five seconds in a mixture of:

Formalin	10%
95% ethyl alcohol	90%

2. Wash in distilled water and dry.

3. Stain with sudan black B solution at room temperature for one-half to one hour. The solution is prepared as follows:

- Sudan black B powder\* to saturation in 70 per cent ethyl alcohol.
- Preferably allow the solution to stand at least twenty-four hours before using.
- If a darker staining is desired, the process can be carried out at 37° Centigrade.

4. Stain with May-Grünwald Giemsa in the following manner:

- Flood the slide with the May-Grünwald mixture and allow to stand for three minutes. This procedure appears to lend greater sharpness and clarity to the Giemsa stain.
- Cover the slide with neutral distilled water and allow to stand for one minute.
- Drain without washing.

\*National Aniline Division, Allied Chemical & Dye Corporation, New York, N. Y.

- d. Cover with dilute Giemsa mixture (one drop of stock solution to 1 c.c. of water) and allow to stand for ten to fifteen minutes.
- e. Wash in distilled water and dry.

This procedure gives a preparation in which erythrocytes are stained slate gray to black. As Sheehan has already noted, these structures are markedly distorted when fixation is omitted but are well preserved when it is used. We believe that leucocytes are also more faithfully preserved after initial fixation. Since no new structural features of erythrocytes are brought out by the sudan technique, they will not be considered further at this time.

Two main sources were used in securing blood for the subsequent studies:

1. Repeated films were made from blood of five normal individuals. These films were usually stained alternately, one with May-Grünwald Giemsa and one with sudan May-Grünwald Giemsa, and the two types of preparation compared by successive studies under the microscope.

2. Films were also prepared from several types of blood dyscrasias\*:

- a. Two cases of secondary anemia.
- b. Two cases of pernicious anemia.
- c. Three cases of acute lymphatic leucemia.
- d. One case of chronic lymphatic leucemia.
- e. Five cases of acute myelogenous leucemia.
- f. Three cases of chronic myelogenous leucemia.
- g. Three cases of acute infectious mononucleosis.
- h. Two cases of pneumonia with eosinophilia.

In using the previously mentioned preparations, no attempt was made at a pathologic study per se, but special attention was directed toward identification of individual leucocytes and examination of their granular content.

#### OBSERVATIONS AND DISCUSSION

*Studies on Normal Blood.*—When one examines blood films stained by sudan black B only, he encounters occasional groups of sharply blackened granules; only by markedly decreasing the field illumination can he determine that these bodies are actually enclosed by the cytoplasm of otherwise unstained granulocytes. Despite the difficulty of using such films, they are sometimes valuable as an aid in examination of individual granules since they are there exposed in maximum contrast, that is, black against a tan, yellow, or colorless background. In this type of preparation one finds that the stained inclusions differ with respect to size, shape, stainability, and distribution in various leucocytes and that these differences are characteristic for the granulocyte cell types.

Successive examinations of May-Grünwald Giemsa and sudan May-Grünwald Giemsa-stained blood films indicate that the two types of preparations differ only with respect to the granular content of their leucocytes. Preliminary fixation and staining with sudan in no wise affect the tinctorial properties of

\*These slides were furnished through the courtesy of Dr. Philip Pizzolato, Department of Pathology, Charity Hospital of Louisiana, New Orleans, La., and the late Dr. C. A. Stewart, Department of Pediatrics, Louisiana State University School of Medicine, New Orleans, La. The diagnoses were made by Dr. Pizzolato.

other cell constituents. For this reason it is possible to use nuclear architecture and color reaction as an aid in identifying various cells. Again, all cytoplasmic granules of nonspecific nature are unaltered by exposure to the sudan dye.

After preparation of a normal blood film by the sudan May-Grünwald Giemsa technique, *neutrophile* granules are well preserved and clearly demarcated from the surrounding cytoplasm. Under these conditions they appear to be of constant size and tinctorial properties, staining slate gray. Any apparent variability in size or color is due to the fact that they tend to collect in groups within the cytoplasm, and the observer is sometimes forced to examine the elements in superimposed state. Such granular complexes may have irregular margins and deeply staining centers. When the granules are isolated within the cell, however, each is spherical in form and sharply demarcated from surrounding cytoplasm.

The total number of neutrophile granules per cell varies considerably even under normal conditions. This variability cannot be correlated with any feature such as the number of nuclear lobes or total cell diameter. When few in number, the granules tend to collect in a zone adjacent to the centrosphere, leaving the cytoplasmic periphery relatively clear. As their number increases more are crowded toward the cytoplasmic membrane. Grouping of individual elements may be encountered in any part of the cytoplasm.

Granules of normal *eosinophile* leucocytes are well demonstrated in sudan May-Grünwald Giemsa-stained blood films. It is common to find eosinophile granules so crowded in the cytoplasm that one is sometimes forced to search for an isolated element. Under favorable conditions, however, each granule is found to consist of a deeply blackened shell and a clear, unstained core. There is a sharp line of demarcation between the two regions. In eosinophiles which have been deeply stained by the acidophilic components of the May-Grünwald and Giemsa mixtures, the sudanophobic cores are pink in color.

The granules of a majority of normal eosinophiles are spherical in form. In a few instances, however, they are shaped into oblate spheroids or plump spindles. This corroborates the findings of Downey<sup>5</sup> and others for corresponding granules in animal leucocytes. Either type of adspherical granule has a sudanophilic shell of rather constant thickness and a sudanophobic core whose form simulates that of the granule as a whole. Especially in spindle-shaped forms there is a marked condensation of sudanophilic material in the poles of the granule; these densely blackened regions gradually merge with the remaining portions of the shell.

*Basophile* leucocytes from normal blood are difficult to study because of their scarcity, but at least one was eventually located in each prepared film. The mast granules appear to be morphologically and tinctorially identical in sudan May-Grünwald Giemsa and May-Grünwald Giemsa-prepared films. As is well known, these metachromatic bodies are colored deeply purple or purplish black with the usual hematologic stain combinations. Since this color is not strikingly different from the gray or blue black imparted to most leucocyte inclusions after exposure to sudan black B, one at first hesitates to state that mast

granules are entirely sudanophobic. Careful studies have been made on films stained with the sudan alone, however, and no basophile granules appear there in stained condition. We shall have occasion to consider them again in our studies of pathologic blood (*vide infra*).

*Agranulocytes* (lymphocytes of all sizes and monocytes) are readily found in normal blood films prepared by the sudan May-Grünwald Giemsa technique. These cells are identical in all particulars with those seen in preparations stained with May-Grünwald Giemsa alone, that is, they are quite unaffected by exposure to sudan black B.

In any adequately stained preparation of normal blood, one is frequently able to find azurophilic granules in both monocytes and lymphocytes. As is well known, these inclusion bodies show considerable variability in size, many ranging downward to the lower limits of microscopic visibility. With the usual hematologic dye combinations they stain in color values from brick red to reddish violet. Exposure to sudan black B appears to alter their tinctorial properties but little. It is possible that they become darker in tone, but this change is not of sufficient degree to preclude the possibility of subjective error.

*Studies on Abnormal Blood.*—No concerted attempt was made in these studies to differentiate the various blood dyscrasias; attention was primarily centered on cell identification and on examination of the cytoplasmic granules in various immature and pathologic leucocytes.

Blood films from several types of *secondary anemia* were studied both after staining by the May-Grünwald Giemsa and sudan May-Grünwald Giemsa techniques. In general, no strikingly new features in their leucocyte granules were revealed by exposure to the sudan stain. In one case there were numerous well-marked toxic neutrophils whose granules were large and unusually basophilic in reaction. These granules were readily identified in the sudan preparations as discrete, densely blackened bodies. It will be remembered that normal neutrophile granules are expected to be slate gray after staining with this dye; thus, toxic alterations in the granules are reflected by an increase in their sudanophilic power.

In all cases of *pernicious anemia* followed in these investigations, it was possible to identify at least a few of the so-called "pernicious anemia neutrophile leucocytes." Their granules are larger than those of normal polymorphonuclear cells and tend to be more acidophilic in reaction. Treatment of these blood films with sudan black B reveals that the pernicious anemia neutrophile granules are but lightly sudanophilic, staining a light gray. Despite this mild chromatophilia, however, they are well demarcated and sharply outlined in the surrounding cytoplasm.

Blood films from four *lymphatic leukemia* cases were carefully studied in an effort to identify accurately as many of the leucocytes as possible. It was found that in both acute and chronic lymphatic leukemia none of the markedly abnormal leucocytes were sudanophilic. Especially in the acute form there appeared greatly increased numbers of lymphocytes and lymphocytoid cells. Some of these pathologic elements have basophilic cytoplasm and large, pale nuclei whose chromatin is laid down in a rather sievelike pattern. Nucleoli may or



may not be present. Comparison of May-Grünwald Giemsa and sudan May-Grünwald Giemsa-stained slides from these cases reveals no differences in the previously mentioned cells. A small number of them are granulated to some degree. The inclusions are irregular in size, distribution, and tinctorial properties, but in all instances they are quite unaffected by exposure to sudan. At least some of these pathologic lymphocytes can be identified as frank stem cells, and it is interesting to note that in our preparations they are invariably sudan-free.

The leucocytes from eight cases of *myelogenous leucemia* showed marked variability of reaction to sudan black B stain. As was found in our lymphatic leucemia studies, none of the stem cells contained sudanophilic granules (Fig. 1); with few possible exceptions, the leucoblasts were also sudan-free. On the other hand, promyelocytes of both the neutrophile and eosinophile series were noted to contain sudanophilic bodies in considerable numbers (Fig. 2).

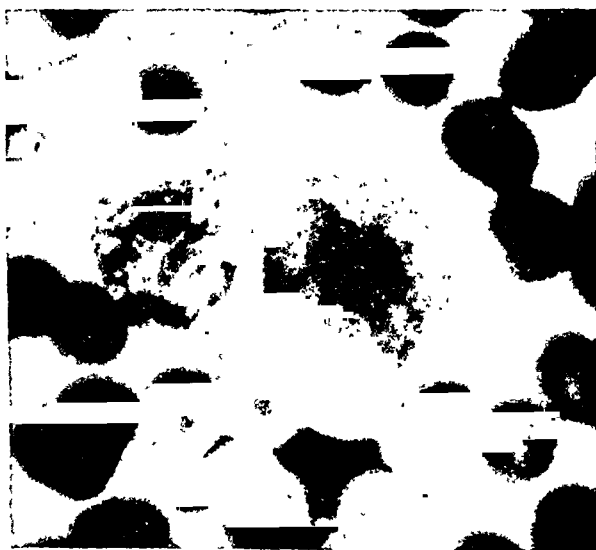


Fig. 1.—Blood film from a case of *myelogenous leucemia*. The cell on the right is an eosinophile metamyelocyte. The cell on the left is a late stem cell. The metamyelocyte contains sharply staining granules which stain with the sudan.

The cell on the right is a late stem cell. The stem cell contains no granules.

The cells shown in Figs. 1 to 6 were found in blood films which had been fixed in formalin-alcohol and stained with Sudan May-Grünwald Giemsa. All are magnified 1640X.

Early neutrophile myelocytes have highly variable numbers of cytoplasmic granules but in all cases are somewhat sudanophilic from the time of their inception. During maturation through the myelocyte and metamyelocyte stages of development, these bodies increase but little in size although their staining power is considerably augmented (Fig. 3). As early as in the myelocyte form, some of the granules appear as slate-gray spheres, but the majority do not stain in this manner until almost complete maturation has been accomplished. Especially in myelocytes one first notes that these granules tend to collect in groups.

Promyelocytes of the eosinophile series likewise contain sudanophilic granules. From the time of their formation many are notably larger in size than their neutrophilic counterparts and can be differentiated from them on this basis alone. With maturation the eosinophile granules become progressively more stainable, but the chromatophilia is confined to their peripheries. In most myelocyte and in a few promyelocyte granules, one can make out the darkly staining shells and nonstaining cores. The sudanophilia of these shells continues to increase until full maturation of the cell has been completed (Fig. 4).



Fig. 2.—In this figure are shown a number of cell types in a film from a case of acute myelogenous leukemia in extremis. At the extreme right is a stem cell; its cytoplasm contains no sudanophilic granules. At the left is a late leucoblast or early promyelocyte, probably of the neutrophilic series. This latter cell is surrounded by a number of promyelocytes and myelocytes of variable size. Note in these cells that the developing definitive granules are densely sudanophilic. The intercellular debris is composed chiefly of cell fragments.

Immature leucocytes of the basophile series are identifiable in films from our cases of myelogenous leukemia. Repeated studies have led us to the conclusion that their granules are never sudanophilic at any stage of development. The mast promyelocyte typically contains granules which stain in some value of gray, both in the May-Grünwald Giemsa and sudan May-Grünwald Giemsa-stained preparations. One might consider that this shade is due to slight sudanophilia, but comparison of the two preparation types fails to show that exposure to sudan causes any change in their tinctorial properties. For this reason we believe that they are stained only by the secondary dye mixture. Progressive basophilia and metachromasia were noted as these granules appear in more mature forms.

One of the cases of myelogenous leukemia deserves special mention in that the first films of the patient's blood were taken when he appeared at the hospital in a sudden clinical crisis. At the time of admission the blood count indicated 125,000 leucocytes per cubic millimeter; a few of these were stem cells and leuco-

blasts. Promyelocytes, myelocytes, and metamyelocytes were numerous and easily identified. During the course of hospitalization, the initially high leucocyte count rapidly subsided and accompanying cytologic changes could be followed from the blood films.

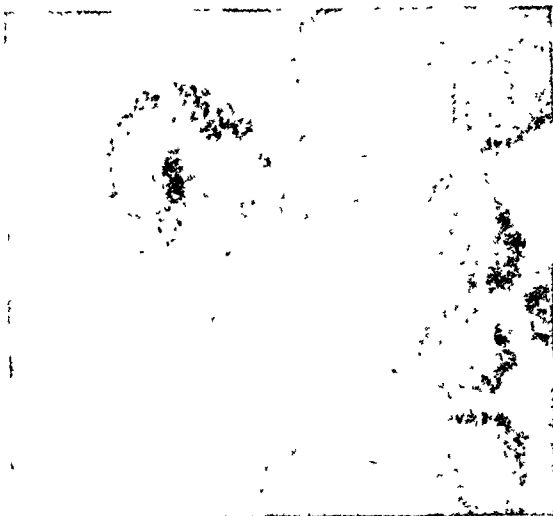


Fig 3.—A late metamyelocyte of the neutrophil series from a case of chronic myelogenous leukemia. Note the small size of the individual granules and that in some places granules are collected into groups.

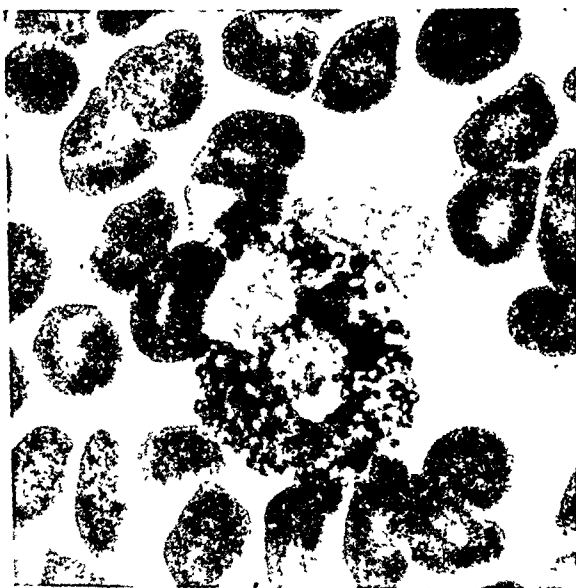


Fig. 4.—A large eosinophile from a case of acute myelogenous leukemia. This cell is almost mature and has numerous well-differentiated cytoplasmic granules. Each granule is composed of a deeply blackened shell and an unstained core region.

As the crisis period neared its close, the great majority of pathologic leucocytes became involved in a degenerative process which culminated in their destruction and disappearance. This process was characterized by nuclear pye-

nosis and cytoplasmic fragmentation. Within a period of about two weeks' time, the patient's blood picture had returned almost to normal and he was discharged from the institution. Periodic re-examinations of the blood revealed a slow but progressive increase in the number of circulating leucocytes and a rise in the number of immature elements.

During the recovery phase briefly described, granules in the degenerating leucocytes showed marked changes in their sudanophilic power. It appears that the advent of cytologic disintegration is accompanied by loss of sudanophilia in such manner that the granules disappear while the cells are still superficially intact. This same process can be seen occasionally in isolated cells from nearly all leucemic blood.

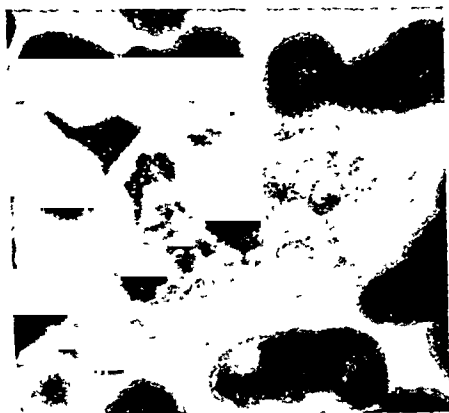


Fig. 5.

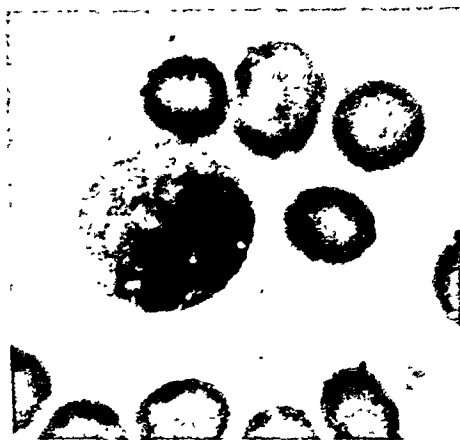


Fig. 6.

Fig. 5.—An eosinophile taken from a case of pneumonia with eosinophilia of unknown etiology. In this instance the definitive granules are neither sharply demarcated nor densely sudanophilic. Many of the granules do not clearly show the core and shell regions.

Fig. 6.—A monocytoid lymphocyte from a case of infectious mononucleosis in the acute phase. Note in the cytoplasm that there are several colorless vacuoles and various granules of inconstant size. These granules are entirely sudanophobic in reaction.

Two cases of *pneumonia with eosinophilia* of unknown etiology were examined in this series. The circulating eosinophiles were, respectively, 18 and 26 per cent of the total white count. Sudan May-Grünwald Giemsa-stained blood films revealed that many of these eosinophiles were markedly abnormal in that they contained many imperfectly formed grayish granules (Fig. 5). In these bodies the shell and core regions were frequently undemonstrable in that the entire granule was weakly and diffusely stained. A few granules appeared to attain full maturity as expressed by normal tinctorial properties.

Blood films obtained from three cases of *acute infectious mononucleosis* in the acute phase were alternately stained by sudan May-Grünwald Giemsa and May-Grünwald Giemsa techniques. As is well known, there is still some doubt as to the identity of certain mononuclear cells which appear in the circulating blood during this disease. Many investigators such as Downey and Stasney<sup>6</sup> now believe that these elements are pathologically modified lymphocytes. In our preparations we were readily able to find the coarse chromatin strands and

masses within their nuclei and to note that these structures were not sharply demarcated from the parachromatin substance. These features have been emphasized already by the previously mentioned authors. The cytoplasm of the pathologic lymphocytes was typically basophilic in reaction, sometimes markedly so, and occasionally contained colorless vacuoles (Fig. 6).

In a few instances the abnormal cells in these cases of acute infectious mononucleosis were noted to contain cytoplasmic granules of considerable size and number. With the usual hematologic stains, these bodies superficially resemble immature neutrophile or even eosinophile granules. Exposure of such cells to sudan black B does not alter their appearance in any respect, that is, they are entirely sudanophobic in reaction. It now seems probable that they are derived from granules of the azurophile series.

From the preceding discussion it can be seen that sudanophilia occurs in many of the same leucocyte forms which are known to have positive oxidase and peroxidase reactions. Despite the many studies on these leucocytic enzymes, there is still considerable divergence of opinion as to the significance of their presence (see Michels<sup>7</sup>). Notwithstanding, many investigators still follow the concept of Naegeli<sup>8</sup> who believed that leucocytes of myeloid and lymphoid origins could be differentiated on the basis of the foregoing reactions. He held that myeloid leucocytes are oxidase and peroxidase positive while those of lymphatic origin are oxidase and peroxidase negative. The same general principle has been applied to immature forms, even to those as far back as the stem-cell stage.

Other investigators such as Weil and Isch-Wall,<sup>9</sup> in contrast, have been unable to determine that oxidase and peroxidase reactions are consistently positive in myeloblasts. Due to the scarcity of stem cells in some bone marrows, much of this investigation has been carried out on pathologic blood. For this reason it is frequently assumed that when these reactions are negative in cells of myeloid origin, the responses are prevented by pathologic changes in the affected cells.

We have stained films of leucemic blood by the methods of Goodpasture<sup>10</sup> and Graham<sup>11</sup> for peroxidase and by the method of Schultze<sup>12</sup> for oxidase. In these preparations we have studied the immature leucocytes in detail and then compared them with corresponding forms stained with sudan May-Grünwald Giemsa and May-Grünwald Giemsa methods. By these comparisons we have found that, with one possible exception, oxidase- and peroxidase-positive granules are consistently sudanophilic. This exception concerns inclusions in stem cells. It is not uncommon, for example, to find peroxidase granules in what appear to be frank stem cells. These granules tend to be irregular in size and variably stained; as such, they might be considered as artifacts formed by precipitation of the dye. The most successful sudan May-Grünwald Giemsa-stained preparations, on the other hand, are precipitate-free, and any stem cells contained therein are also free of blackened granules. From these observations it would seem that the so-called oxidase- and peroxidase-positive granules are not always sudanophilic; there is still some doubt, however, as to interpretation of the oxidase and peroxidase positive reaction.

With the possible exception of those in the myeloblast, all leucocyte granules which are consistently oxidase and peroxidase positive are also sudanophilic and stain blue black or black. Because of the high degree of contrast so effected, the granules can be more easily studied. Again, the sudan-in-alcohol solution is stable over considerable periods of time; this makes frequent solution preparation unnecessary. For these reasons it is believed that the sudan May-Grünwald Giemsa technique may be profitably substituted for various oxidase and peroxidase staining methods.

Especially in three of our cases of myelogenous leucemia there were found considerable numbers of atypical granules in the various immature leucocytes. After preparation with May-Grünwald Giemsa, these granules are amphophilic in reaction and stain reddish blue or reddish violet. On the basis of their tinctorial reaction, then, they may be considered as modified azurophilic granules. In films stained with sudan May-Grünwald Giemsa, they are readily identified as bodies identical with those previously described, both as to size and color. This means that the granules in question are entirely sudanophobic. They may be found in medium and large lymphocytes, monocytes, and occasionally in granulocytes as well. Most of them are smaller in size than definitive leucocyte granules, and some are so small as to approach the lower limits of microscopic visibility. Due to the fact that with one exception, the basophile, definitive granules are consistently sudanophilic and nonspecific granules sudanophobic in reaction, the sudan technique should prove useful in differentiating the two types of inclusions in leucocytes.

As far as is now known, sudan black B is a lipid-specific stain. If this is true, definitive neutrophile and eosinophile granules have a lipid component which appears at their inception and is progressively augmented during their maturation. It is possible that all these granules do not contain exactly the same lipid substance, since some (that is, neutrophile granules) are stained in slate gray while others (eosinophile granules) are deeply blackened. The function of the lipid component is still entirely unknown, but it is invariably found in the normal definitive granule. Pathologic forms, on the other hand, are to some extent deficient in lipid content, either in quantity or in distribution within the granule.

#### SUMMARY AND CONCLUSIONS

Dried films of normal and abnormal blood were stained with sudan black B and then treated with the May-Grünwald Giemsa dye combination. The first stain was found to blacken specifically the definitive neutrophile and eosinophile leucocyte granules and to leave all other cell parts unaffected. Architectural features of these cells, such as nuclear pattern, can be stained by a proved method (May-Grünwald Giemsa) and used as an aid in cell identification. Similarly, the hyaloplasm is also differentially colored so that it serves as an efficient contrast medium for the blackened granules.

After exposure to the sudan stain, neutrophile granules appear as small, spherical slate-gray bodies. They are typically collected into groups within the cytoplasm.

Eosinophile granules are larger in size than the preceding type and are spherical or spheroidal in form. They are composed of a peripheral, deeply sudanophilic shell and a central sudanophobic core. These two regions are sharply demarcated from each other.

Basophile granules are found to be sudanophobic in reaction, as are also azurophile elements.

The granular content of some immature pathologic leucocytes is discussed briefly, especially with reference to the acquisition of sudanophilia during maturation.

Several types of abnormal granules (from pathologic blood) are considered. It is noted that sudan black B is useful in differentiating certain specific (sudanophilic) from nonspecific (sudanophobic) granules, especially when the two forms stain similarly with the usual hematologic dye mixtures.

#### REFERENCES

1. Lison, L.: Sur de nouveau colorants histologiques spécifiques des lipides, *Compt. rend. Soc. de biol.* 115: 202, 1934.
2. Hartman, T. L.: The Use of Sudan Black B as a Bacterial Fat Stain, *Stain Technol.* 15: 23, 1940.
3. Burdon, K. L., Stokes, J. C., and Kimbrough, C. E.: Studies of the Common Aerobic Spore-forming Bacilli. I. Staining for Fat With Sudan Black B-Safranin, *J. Bact.* 43: 717, 1942.
4. Sheehan, H. L.: Staining of Leucocyte Granules by Sudan Black B, *J. Path. & Bact.* 49: 580, 1939.
5. Downey, Hal.: The Origin and Development of Eosinophil Leucocytes and of Hematogenous Mast Cells in the Bone Marrow of the Adult Guinea Pig, *Fol. Hematol. Archiv.* 19: 148, 1915.
6. Downey, Hal, and Stasney, J.: Infectious Mononucleosis. II. Hematologic Studies, *J. A. M. A.* 105: 764, 1935.
7. Michels, N. A.: The Mast Cells, in Downey, Hal (editor): *Handbook of Hematology*, New York, 1938, Paul B. Hoeber, Inc., Vol. 1, Section IV, p. 231.
8. Naegeli, O.: *Blutkrankheiten und Blutdiagnostik*, Lehrbuch der klinischen Hämatologie, ed. 5, Berlin, 1931, Julius Springer.
9. Weil, P. E., and Isch-Wall, P.: Les leucémies à tumeurs, *Sang* 5: 513, 1931.
10. Goodpasture, E. W.: Peroxidase Reaction With Sodium Nitroprusside and Benzidine in Blood Smears and Tissues, *J. LAB. & CLIN. MED.* 4: 442, 1919.
11. Graham, G. S.: Benzidine as Peroxidase Reagent, *J. Med. Research* 39: 15, 1918.
12. Schultze, W. H.: Die Oxydaseraktion an Gewebsschnitten und ihre Bedeutung für die Pathologie. Zugeich ein Beitrag zur Differential diagnose der Leukanamien, *Beitr. z. path. Anat. u. z. allg. Path.* 45: 127, 1909.

# A STUDY OF THE STERNAL MARROW AND PERIPHERAL BLOOD OF FIFTY-FIVE PATIENTS WITH PLASMA CELL MYELOMA

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**P**LASMA cell myeloma is a chronic progressive disease which is characterized by multiple osteolytic plasma cell tumors of bone and diffuse proliferation of plasma cells within the marrow. The malignant cells have a predilection for the bones which are most active in blood cell formation, such as the sternum, ribs, vertebral bodies, bones of shoulder girdle and pelvis, skull, and upper ends of the femora and humeri. Infiltration of plasma cells takes place early in the course of the disease, and aspiration of material from the sternal marrow usually reveals significant changes.

It is the purpose of this paper to summarize the cytologic characteristics of the bone marrow and peripheral blood in plasma cell myeloma, as revealed in the published literature and in marrow and blood films of fifty-five patients with the disease observed at Cleveland Clinic. The diagnosis was established by combined clinical, laboratory, radiologic, and marrow findings. Patients with unsatisfactory marrow preparations, with inadequate follow-up, and in whom the diagnosis was doubtful were excluded. Patients with an increase in plasma cells in the peripheral blood (plasma cell leukemia) also were included in the series.

The sternal marrow was collected by biopsy from one patient and from the others by inserting a needle into the marrow space, aspirating from 0.1 to 0.2 c.c. of fluid and making films directly, using the coverslip technique. The preparations were stained with Wright's stain.

## BONE MARROW

The literature relating to the plasma cell has been well summarized by Maximow<sup>1</sup> and Michels,<sup>2</sup> as have the essential clinical and pathologic features of plasma cell myeloma by Geschickter and Copeland.<sup>3</sup> Descriptions of the bone marrow films in plasma cell myeloma and the value of the procedure in diagnosis and differential diagnosis have been presented by Vogel and associates,<sup>4</sup> Rosenthal and Vogel,<sup>5</sup> Scott,<sup>6</sup> Weil and Perlès,<sup>7</sup> Doan,<sup>8</sup> Haden and Rumsey,<sup>9</sup> Beizer and co-workers,<sup>10</sup> and others.

The number of plasma cells in bone marrow preparations from patients with plasma cell myeloma is variable. This is explained by the fact that the tumors tend to be patchy in distribution and there are varying degrees of dilution with peripheral blood. The sternal marrow films occasionally fail to reveal an increase in plasma cells,<sup>10</sup> but in the majority of cases there is a significant increase. The percentage of plasma cells in the bone marrow films of our series was

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determined in each patient by noting the number while making a differential count of 300 or more nucleated cells. The lowest percentage of plasma cells was 4 and the highest 90. The percentage of plasma cells was greater than 10 in forty-eight of the cases. The frequency distribution of cases according to the percentage of plasma cells is given in Fig. 1. In one patient with plasmocytoma of the clavicle, not included in this series, the percentage of plasma cells was 3.

In normal bone marrow plasma cells are always demonstrable, but their number is less than 1 per cent. In 322 unselected bone marrow films from patients with a clinical diagnosis other than plasma cell myeloma, the plasma cells were less than 1 per cent in 129 cases and less than 4 per cent in 315.

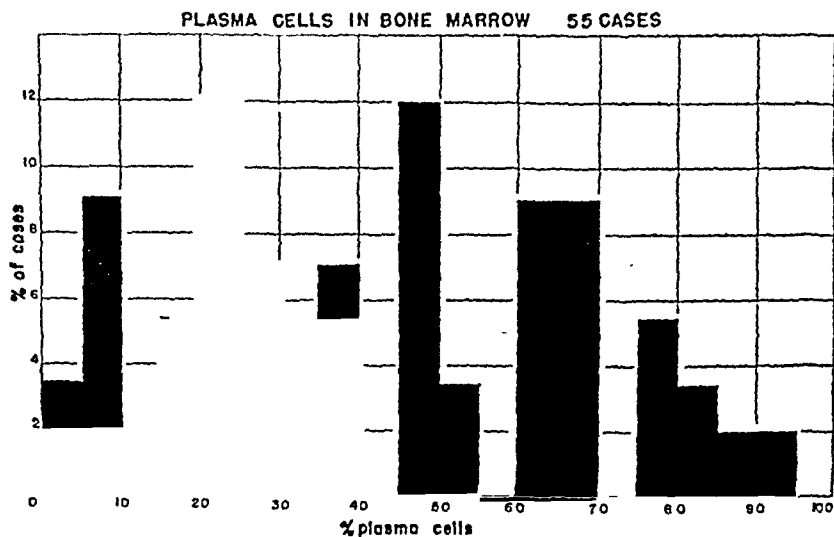


Fig. 1.—Frequency distribution of the percentage of plasma cells in the bone marrow from fifty-five patients with plasma cell myeloma.

In seven patients the number varied between 4 and 9 per cent. In three of these the diagnosis was not definitely established, and the possibility of plasma cell myeloma was not excluded. The final diagnoses in the other four patients were carcinoma, agranulocytosis, aplastic anemia, and hypochromic anemia due to bleeding fibroids. Plasma cell increase in the bone marrow has been described in chronic infections, metastatic malignancy, Hodgkin's disease, the leucemias, agranulocytosis, and aplastic anemia.

An analysis of our patients with plasma cell myeloma did not reveal any significant correlation between the percentage of plasma cells in the marrow and the duration of symptoms, the degree of anemia, the number of plasma cells in the peripheral blood, or the number of grams of total protein or globulin in the blood serum. Films with the higher percentages of plasma cells tended to have more cytoplasmic debris, but this likewise varied in different patients and in different slides from the same patient.

Plasma cells of all stages of maturation from the earliest undifferentiated cells to the more mature forms were encountered. The cells were classified on

the basis of nuclear, nucleolar, and cytoplasmic characteristics into plasmoblasts, early plasma cells, and mature plasma cells (Fig. 2).

The plasmoblast has the characteristics of other primitive cells. This cell is larger than the mature plasma cell and has a relatively large round nucleus and a light blue, unevenly staining cytoplasm. The nucleus is round and has a delicate chromatin pattern and one to four nucleoli. The nucleus tends to be eccentric; the nucleolus is usually single, very large, and well defined. The cytoplasmic margin is often irregular, and transition forms can be found between this cell and the typical plasma cells.

The early or intermediate plasma cell has a less distinct nucleolus than the plasmoblast, the nuclear chromatin is more compact, the cytoplasm stains a darker blue, the perinuclear clear zone is more distinct, and the amount of cytoplasm in relation to the nucleus is greater. The cytoplasm stains unevenly and the shape is irregular.

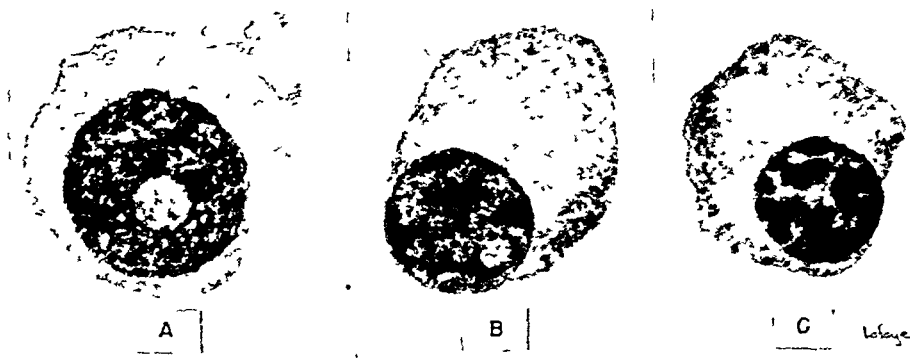


Fig 2—A, Plasmoblast; B, Early plasma cell; C, Mature plasma cell.

The mature plasma cells seen in plasma cell myeloma resemble those seen in normal bone marrow and in numerous other conditions. The cell is usually oval and is slightly larger than the neutrophilic leucocyte. The nucleus is relatively small, eccentric, round, and has a pachychromatic structure. The so-called cart wheel nucleus, which is often described as being typical of this cell in tissue sections, is seldom seen in dried blood films stained with Wright's stain. There is a prominent, relatively unstained area near the nucleus. The cytoplasm stains an intense blue and often has a greenish cast. In some of the cells the cytoplasm may stain so darkly that no structure is visible, but in the majority of cells the cytoplasm has a fine, interlacing linear structure or there are ill-defined amorphous bluish flecks. Plasma cells are often spoken of as having a granular cytoplasm, but well-defined granules such as are observed in neutrophilic and eosinophilic myeloid cells are not seen. In some cells the cytoplasm appears foamy or there may be multiple vacuoles of various sizes. A collarlike layer of clear hyaline material may be seen sometimes at the periphery of the cell, or the outer portion of the cell may be light and relatively structureless with



Fig. 3.—Drawings showing plasma cell variants in bone marrow films, plasma cell myeloma. A, B, and C, Early plasma cells with cytoplasmic streamers; D, plasma cell with plicate nuclear chromatin and hyaline periphery; E, mature plasma cell with cytoplasmic extensions; F, plasma cell with two nuclei.

hairlike bluish strands extending into this area from the darker stained central portion of the cell (Fig. 3D). Homogenous spherical bodies, usually acidophilic but staining various shades of red to blue (Russell bodies), are occasionally demonstrable (Fig. 4C). The mature plasma cell and its progenitors react negatively with peroxidase stains.

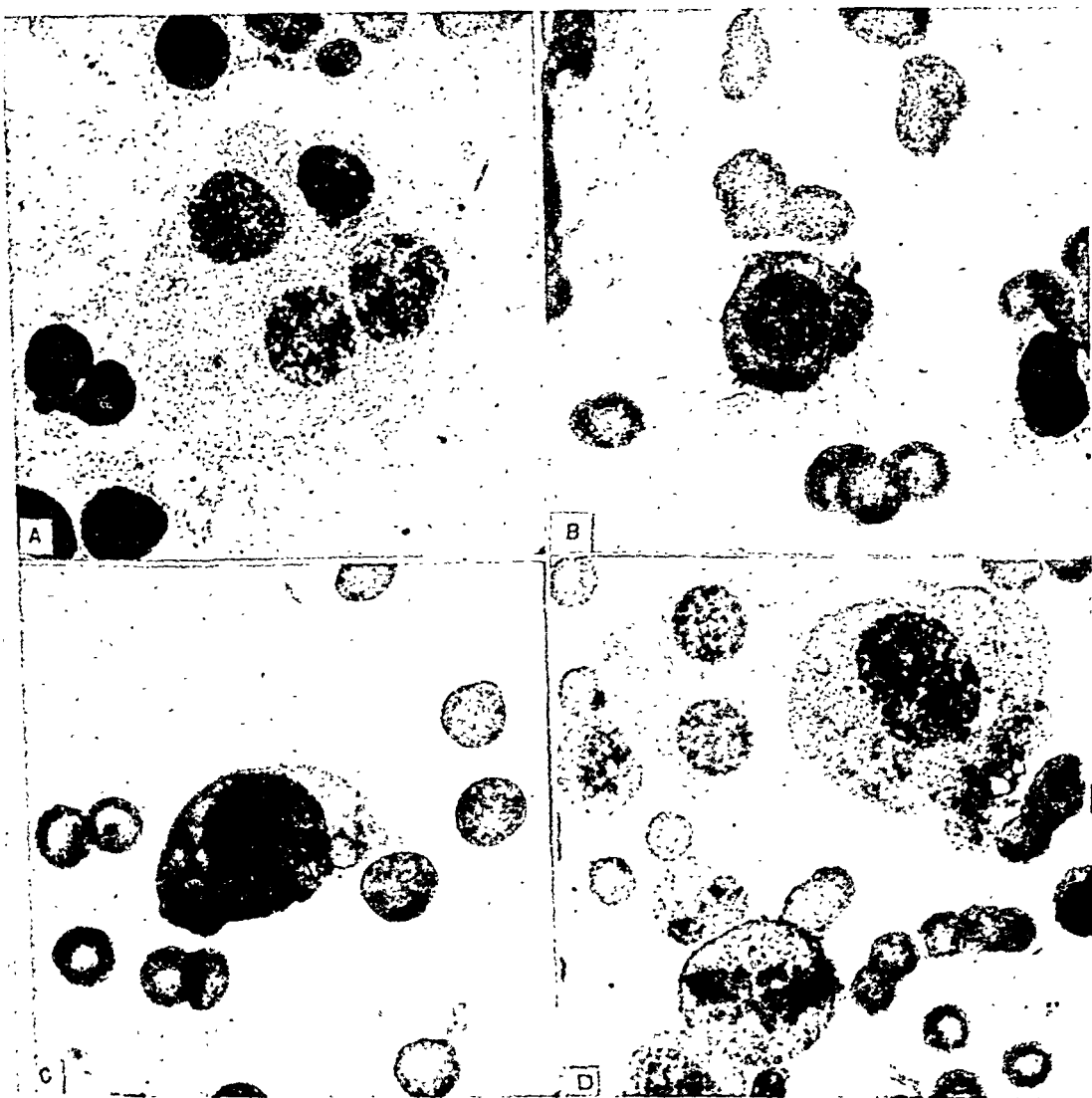


Fig. 4.—Photomicrographs of plasma cells in bone marrow, plasma cell myeloma. A, Multinucleated giant cell with vacuoles in cytoplasm and ill-defined margin, cytoplasmic debris; B, plasma cell showing hairlike cytoplasmic streamers; C, plasma cell with eosinophilic globules (Russell bodies); D, plasma cells: one with two nuclei, giant form with vacuoles, intermediate nucleus, and cytoplasmic projections, and one in mitosis. Rouleaux formation.

The distribution of plasmoblasts, early plasma cells, mature plasma cells, and atypical forms in each of our patients was determined by analyzing 100 plasma cells. The results are graphically represented in Fig. 5. It is noted that

plasmoblasts are present in varying numbers in forty-three cases, that the early forms are found in all films, and that atypical cells are present in significant numbers in the majority of cases. Cells showing degenerative changes were included with the atypical cells.

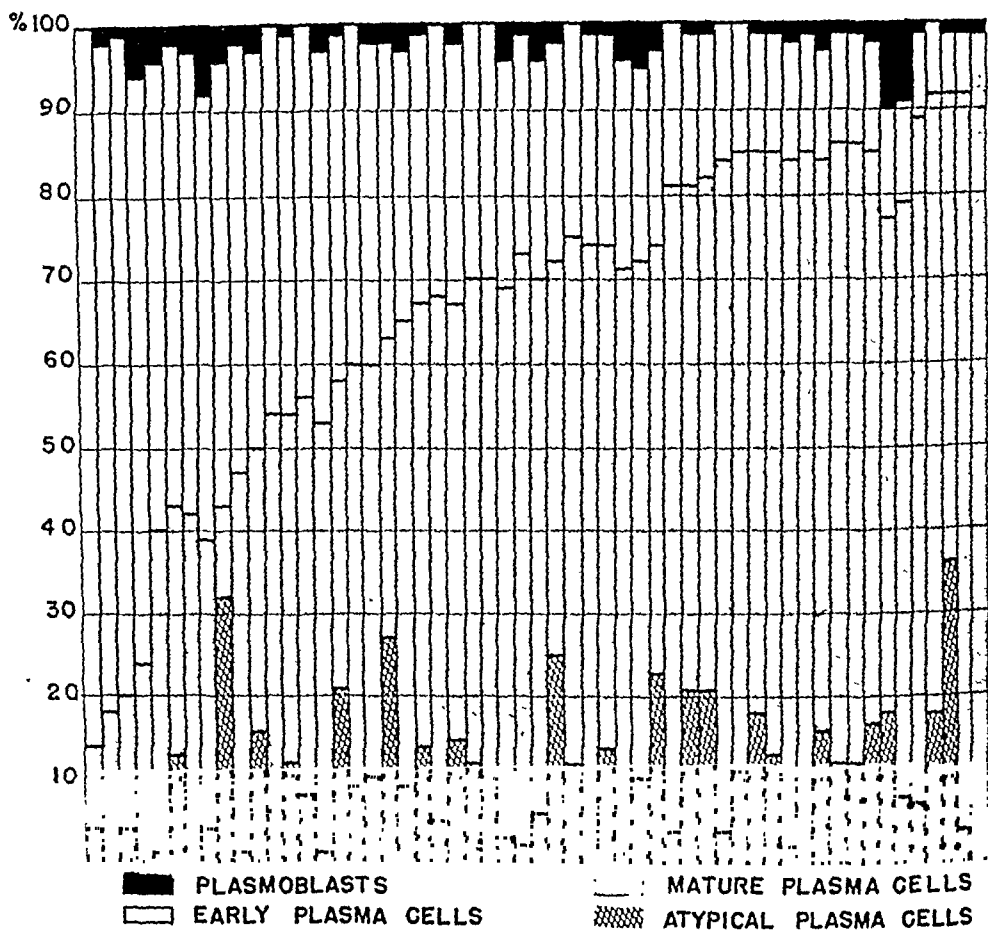


Fig. 5.—The distribution of plasmoblasts, early plasma cells, mature plasma cells, and atypical forms in fifty-five patients with plasma cell myeloma.

In plasma cell myeloma there is evidence of increased and abnormal cell division. Mitotic figures are usually demonstrable and cells with multiple nuclei common (Fig. 4A, D). The number of nuclei in a given cell varies from two to six. The nuclei are characteristically separated from each other, are round or oval, and are of the same size; however, indented or lobulated nuclei, nuclei of different sizes, and nuclear fragments are sometimes seen.

Plasma cells tend to appear in pairs or in small groups in normal marrow. In plasma cell myeloma there may be large nests of contiguous cells which are often flattened or polyhedral in shape.

One of the characteristics of the plasma cell as seen in normal bone marrow and as an exaggerated and prominent feature in plasma cell myeloma is the marked variation in shape of the cells and the tendency for the cells to be frayed and ragged in outline. These cytoplasmic projections may be short and blunt or long, slender, and hairlike (Figs. 3 and 4). Irregularities and fragmentation of the cytoplasm have been noted by various authors,<sup>1, 2, 11, 12</sup> but little emphasis has been placed upon this feature in descriptions of cells or in illustrations. The cells that have ill-defined margins and tapering tortuous filamentous streamers constitute from 10 to 50 per cent of the plasma cells. The streamers are characteristic of the cells at all stages of maturation but are most numerous in the earlier cells. It is apparent from the study of bone marrow films from patients with plasma cell myeloma and from nonmyelomatous conditions that plasma cells are fragile, that their margins are readily torn and smudged, and that partial disintegration is characterized by hyaline and filamentous cytoplasmic protrusions which merge in a nebulous manner with the background. In many preparations it is possible to show continuity between the cells and the cytoplasmic debris (Fig. 4A). It is probable that the increased and abnormal serum proteins found in association with plasma cell myeloma have their origin in plasma cells and that the extracellular structures seen in the marrow films are visible manifestations of these proteins at the site of their formation.

Plasma cells with cytoplasmic streamers are less striking in tissue sections or in marrow films made by techniques which require mechanical manipulation and delay than in films made directly. When 1 to 5 c.c. of marrow are withdrawn, an anticoagulant is added, the material centrifuged, and the supernatant cellular layer mixed before spreads are made, the more fragile cells are destroyed, and the remaining cells tend to assume a spherical form.

The plasma cells in plasma cell myeloma vary greatly in size as well as in shape. The predominant cells are larger than normal, and giant forms are often found (Fig. 4A and D).

Acidophilic globules or Russell bodies (Fig. 4C) were present in the cytoplasm of the plasma cells of several of our patients and have been observed by one of us (L. W. D.) as a prominent feature in a single case of a patient with plasma cell leukemia not included in this series, but these structures were not demonstrable in the majority of cases. No cells were observed which contained in their cytoplasm red cells, pigment, or other identifiable particulate matter.

The clear zone which is characteristic of normal plasma cells is not adjacent to the nucleus in individual cells in plasma cell myeloma.

There was a tendency toward a left shift in the myeloid, erythroid, and megakaryocytic elements, but there were no gross alterations in the maturation pattern of these cells. Lymphocytes constituted above 15 per cent in fourteen of the fifty-five cases, and the average lymphocyte percentage was 12. Eosinophiles were demonstrable in most films, but in no cases were they more than 4 per cent. Histiocytes were infrequent.

Rouleaux formation, cellular debris, and bluish background were striking in the majority of cases.

## THE PERIPHERAL BLOOD

The plasma cells in the peripheral blood of fifty-three patients were estimated by noting the number per 1,000 leucocytes. No plasma cells were found in twenty-six cases, and 0.1 to 0.9 per cent were observed in nineteen cases. The number was 1 to 3 per cent in four cases and 4, 7, 21, and 46 per cent in individual cases.

The types of plasma cells found in the peripheral blood were analyzed according to eccentricity of nucleus, perinuclear clear zone, maturity of nucleus, presence of nucleoli, vacuoles in cytoplasm, and cytoplasmic streamers. The conclusions drawn were that cells comparable to those found in the bone marrow could be found in the peripheral blood, but that the peripheral blood cells as a class were more mature and tended to have less irregularity in shape and fewer cytoplasmic tags. Occasionally cells with distinct nucleoli were observed. Many cells were found which were difficult to distinguish morphologically from immature and atypical lymphocytes.

The differential count in the peripheral blood was not remarkable. There was a tendency toward a slight left shift in myeloid elements with occasional myelocytes and metamyelocytes in a few of the cases. There were occasional nucleated red cells. The platelets were normal in most of the cases but tended to be decreased in the terminal stages or in association with leucemic states.

Morissette and Watkins<sup>13</sup> considered that the blood film was of aid in the diagnosis of plasma cell myeloma and stressed the importance of greasiness of the film, anemia, excessive rouleaux formation, immature erythrocytes and leucocytes, lymphocytosis, eosinophilia, and the presence of "myeloma" and atypical plasma cells.

The suspicion of plasma cell myeloma has occasionally been aroused in our laboratory by the finding of exaggerated rouleaux formation and by an increase in plasma cells, including early and atypical forms. Rouleaux formation, on the other hand, is a common and nonspecific finding. Atypical and early cell types and plasma cells in small numbers are often observed in nonmyelomatous conditions.

## DISCUSSION

Individual atypical plasma cells closely resemble and may be impossible to distinguish from individual erythroblasts, early myeloid cells, and lymphocytes, but the cell maturation sequences of the various cell types are different, and there is little difficulty in distinguishing the plasma cells as a group from myeloid, erythroid, and lymphoid groups. Cells of the myeloid series have definite and well-defined granules, whereas plasma cells have a mottled appearance or linear cytoplasmic structure. Myeloid cells as a class have a smooth margin and seldom have a prominent unstained area near the nucleus, whereas plasma cells have irregular shapes and a clear zone adjacent to the nucleus. The peroxidase stain may be helpful in making the distinction between cells of the myeloid and plasma cell types. Erythroblasts and atypical lymphocytes

may have cytoplasm which stains darkly, but these cells as a rule have a relatively large nucleus, the clear zone is not prominent, there are not likely to be vacuoles or spongy cytoplasm, and there are seldom cytoplasmic streamers extending from the periphery of the cell.

All of the patients with myeloma in our series were of the plasma cell type. Early myeloid cells were found in many films; however, this was not accepted as evidence for the myeloid type of myeloma, for there were no demonstrable transitions between the myeloid cells and plasma cells. Myeloid cells are normal bone marrow constituents, and these cells are to be expected in varying numbers in any malignancy involving the marrow. In our opinion, to call a plasma cell myeloma a myeloid myeloma because there are myeloid cells also present is as illogical as it would be to call a myeloid leucemia a plasma cell leucemia because there are plasma cells demonstrable.

In the literature there are frequent references to the "myeloma cell" as if there were one cell type distinctive of the disease. The descriptions of the so-called "myeloma cell" vary in minor details but in general are those described previously as plasmoblasts, the essential features being an early cell with a round eccentric nucleus containing a large and well-defined nucleolus.<sup>14, 15</sup> The demonstration of plasmoblasts and the presence of large numbers of these very early cells is of great value in the diagnosis of malignancy of the plasma cell type, but there is little justification for picking out this one cell in a sequence and giving to it a nondescriptive and nonspecific name.

It was possible to find in the bone marrow preparations from patients with plasma cell myeloma transitions between the plasmoblasts and undifferentiated fixed tissue cells of the reticulum type. Our observations lend support to the thesis held by many workers that the plasma cells arise from primitive reticulum cells and exist as a specific and distinctive strain of cells.

It is apparent that there is no sharp line of demarcation between the solitary plasmocytoma, plasma cell myeloma, and plasma cell leucemia, for the types of cells found in these three conditions are similar, there are all degrees of variation, and the same patient may exhibit all three features of the disease in different stages of the process.

The bone marrow examination is useful not only in confirming or making the diagnosis of plasma cell myeloma but in differentiating this disease from other diseases. The finding of a normal or only slightly increased number of plasma cells which are of a mature type is of great value in ruling out plasma cell myeloma. In doubtful cases the repetition of the marrow examination at a later date or puncture at a different level of the sternum is indicated.

#### SUMMARY

Films made from the sternal marrow of fifty-five patients with plasma cell myeloma revealed:

1. Increased cellularity.
2. Relative and absolute increase in plasma cells, 4 to 90 per cent, with more than 10 per cent in forty-eight cases.



3. Increased number of plasmoblasts and early plasma cells and plasma cells in mitosis.
4. Grouped plasma cells.
5. Nuclear abnormalities in plasma cells:
  - (a) Multiple nuclei
  - (b) Nuclear fragments
  - (c) Indented and lobulated nuclei
6. Cytoplasmic abnormalities in plasma cells:
  - (a) Displacement or absence of relatively unstained areas in some of the cells
  - (b) Variations in size with tendency toward large forms
  - (c) Marked irregularities in shape with blunt and hairlike cytoplasmic projections and tortuous and ragged cytoplasmic streamers; cytoplasmic debris
7. Relative and absolute decrease in myeloid, erythroid, and megakaryocytic elements with a tendency toward immaturity. Slight lymphocytosis. Eosinophiles usually demonstrable.
8. Rouleaux formation.

Plasma cells with eosinophilic globules (Russell bodies) were demonstrable in several cases but were not observed in the majority.

No plasma cells were found in the peripheral blood films while 1,000 leucocytes were counted in twenty-six of fifty-three cases; less than 1 per cent were found in nineteen, 1 to 3 per cent in four, and more than 4 per cent in four. Plasma cells in the peripheral blood were similar to those found in the bone marrow but tended to be more mature and less irregular in shape.

No instances of myeloid, erythroid, or lymphocytic types of multiple myeloma were observed.

The cells described in the literature as "myeloma cells" have morphologic characteristics of early plasma cells. The term "myeloma cell" should be discontinued, for it is nonspecific and nondescriptive.

This study lends support to the following theses which have been proposed by others:

1. Plasma cells arise from reticulum cells.
2. Plasma cells constitute a distinct strain of cells morphologically different from myeloid, lymphoid, and erythroid types.
3. There is no sharp line of demarcation between solitary plasmocytoma, plasma cell myeloma (multiple plasmocytoma), and plasma cell leukemia.

#### REFERENCES

1. Maximow, A. A.: Plasma Cells, Special Cytology, New York, 1928, Paul B. Hoeber, Inc., pp. 353-367.
2. Michels, N. A.: Plasma Cell, Arch. Path. 11: 775-793, 1931.
3. Geschickter, C. F., and Copeland, M. M.: Tumors of Bone, New York: American Journal of Cancer, 1931, pp. 430-475.
4. Vogel, P., Erf, L. A., and Rosenthal, N.: Hematological Observations on Bone Marrow Obtained by Sternal Puncture, Am. J. Clin. Path. 7: 498-515, 1937.

5. Rosenthal, N., and Vogel, P.: Value of Sternal Puncture in Diagnosis of Multiple Myeloma, *J. Mt. Sinai Hosp.* 4: 1001-1019, 1938.
6. Scott, B. B.: Sternal Puncture in Diagnosis of Diseases of Blood-Forming Organs, *Quart. J. Med.* 8: 127-172, 1939.
7. Weil, P. E., and Perlès, S.: *La ponction sternale. Procédé de diagnostic cystologique*, Paris, 1938, Masson & Cie.
8. Doan, C. A.: Bone Marrow, Paul B. Hoeber, Inc., *Handbook of Hematology* (Downey, H.), New York, 1938, 3: 1926-1927.
9. Haden, E. L., and Rumsey, J. M.: Multiple Myeloma, or Myelomatosis, *M. Clin. North America* 24: 369-380, 1940.
10. Beizer, L. H., Hall, B. E., and Giffen, H. Z.: Diagnosis of Multiple Myeloma by Sternal Aspiration, *Am. J. M. Sc.* 203: 829-836, 1942.
11. Christian, H. A.: Multiple Myeloma: a Histological Comparison of Six Cases, *J. Exper. Med.* 9: 325-351, 1907.
12. Osgood, E. E., and Hunter, W. C.: Plasma Cell Leukemia, *Folia haemat.* 52: 369-383, 1934.
13. Morissette, L., and Watkins, C. H.: Multiple Myeloma; Diagnostic Value of Blood Smear, *Proc. Staff Meet., Mayo Clin.* 17: 433-440, 1942.
14. Hansen, A. T.: Case of Myelomatosis With Diffuse Plasma Cell Infiltration of Lymph Nodes, Liver, Spleen, Kidneys, and Lungs, *Acta med. Scandinav.* 115: 514-523, 1943.
15. Gormsen, H.: *Knoglemarvsundersogelser*, Thesis for Doctorate, Copenhagen, 1942.

## THE INFLUENCE OF HEAT AND FORMALIN UPON THE RH AGGLUTINOGEN

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**D**URING the course of a series of experiments on the Rh factor, differences between the Rh agglutininogen and the A and B agglutinogens were noted in regard to their sensitivity against heat. It was observed that red cells suspended in saline and heated to 56° C. lost their agglutinability by anti-Rh serum, whereas the agglutinability of these red cells by anti-A and anti-B sera was apparently not affected. To corroborate these chance observations the following experiments were performed.

Freshly drawn Rh-positive cells of good agglutinability were washed three times and then resuspended in normal saline to a 2½ per cent concentration. From this stock suspension 2 c.c. each were then transferred into a series of small tubes equal in regard to size, lumen, and thickness of wall. The tubes were stoppered with corks and placed in a water bath at 56° C. for periods ranging from five to twenty minutes; after heating, the tubes were cooled immediately. The red cells were then tested in the usual way for their agglutinability by adding 2 drops of the cell suspension to 2 drops of undiluted serum. After shaking, the mixtures were placed in the incubator for one-half hour and centrifugalized for one minute at a speed of 1,200 r.p.m. The results were read with the help of a magnifying glass.

It is shown in Table I that, increasing with the period of heating, the agglutination by anti-Rh serum is diminished or rendered negative, whereas the agglutination by anti-A and anti-B sera remains unchanged, that is, 4 plus.

Different strains of red cells behaved differently, in so far as some were more affected by the heating process others less.

Since each experiment was finished on the same day that the blood was drawn, the differences between the various strains cannot be attributed to an aging process of the red cells.

In a second series of experiments the reactivity of heated red cells was tested against anti-Rh agglutinating serum, anti-Rh blocking serum and anti-A, anti-B, anti-M, and anti-N sera. The technical procedure was the same as has been described, with the exception that the washed red cells were resuspended in saline as well as in 30 per cent albumin made from bovine plasma. This was necessary for the test with blocking sera.

Given in Table II is an example of such an experiment. It shows that after five minutes heating the red cells become inagglutinable by anti-Rh agglutinating as well as anti-Rh blocking serum, whereas the agglutination by anti-B and anti-N sera remains unaffected after twenty minutes, is slightly diminished after one hour, and is still present, though weakened, after two hours of heating.

From the Laboratories of the Jewish General Hospital.  
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TABLE I

TESTED AGAINST SERUM	MINUTES KEPT AT 56° C.				UNHEATED CONTROL
	5	10	15	20	
	<i>Red Cells, Group A</i>				
Anti-Rh <sub>0</sub>	-	-	-	-	4+
Anti-A	4+	4+	4+	4+	4+
	<i>Red Cells, Group A</i>				
Anti-Rh <sub>0</sub>	4+	2+	1+	-	4+
Anti-A	4+	4+	4+	4+	4+
	<i>Red Cells, Group B</i>				
Anti-Rh <sub>0</sub>	3+	2+	2+	1+	4+
Anti-B	4+	4+	4+	4+	4+

4+ = Red cells clumped to a disk, which is not dissolved by slight shaking.

- = No agglutination.

TABLE II. RED CELLS, GROUP B

TESTED AGAINST SERUM	RED CELLS SUSPENDED IN	MINUTES KEPT AT 56° C.						CONTROL UNHEATED
		3	5	10	20	60	120	
Anti-Rh <sub>0</sub> (agglutinating)	Saline	3+	-	-	-	.	.	4+
	Albumin	3+	-	-	-	.	.	4+
Anti-Rh <sub>0</sub> (blocking)	Saline	-	-	-	-	.	.	-
	Albumin	2+	-	-	-	.	.	4+
Anti-B	Saline	4+	4+	4+	4+	.	.	4+
	Albumin	4+	4+	4+	4+	3+	2+	4+
Anti-N	Saline	4+	4+	4+	4+	.	.	4+
	Albumin	4+	4+	4+	4+	3+	2+	4+

TABLE III

TESTED AGAINST SERUM	FORMALIN CONCENTRATION PER CENT										CONTROL
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	
	<i>Red Cells, Group AB</i>										
Anti-Rh <sub>0</sub>	4+	3+	1+	-	-	-	-	-	-	-	4+
Anti-B	4+	4+	4+	4+	4+	4+	1+	1+	1+	-	4+
Anti-A	4+	4+	4+	4+	4+	4+	2+	1+	1+	-	4+
	<i>Red Cells, Group A</i>										
Anti-Rh <sub>0</sub>	2+	1+	±	-	-	-	-	-	-	-	4+
Anti-A	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+

Heating periods of one hour and longer were possible only with red cells suspended in albumin. Red cells suspended in saline were almost completely hemolyzed after twenty minutes' heating, at which time the cells in albumin did not show any signs of hemolysis. The fact that the red cells lost their reactivity with agglutinating as well as with blocking serum supports Wiener's conception that all Rh antibodies (univalent and bivalent) act upon the same cell receptor.

In a third series of experiments the influence of chemicals upon the Rh factor compared to the A and B factors was studied. Out of several substances which had been tested, the only one which produced a selective effect was formalin.

Red cells washed three times were resuspended in normal saline containing different amounts of formalin, the final concentration of the latter ranging from 0.15 to 1.0 per cent. The formalinized red cell suspensions were kept in the refrigerator overnight; the next morning the agglutinability was tested.

Two examples of the findings are given in Table III. From these it can be seen that formalin in low concentrations inhibits the agglutination by anti-Rh serum. The agglutination by anti-A and anti-B sera is not or is to a lesser

degree affected. Concentrations higher than 1 per cent have an inhibiting effect upon the agglutination by anti-A and anti-B sera as well.

Since increasing the length of heating time has a hemolyzing effect upon red cells suspended in saline, a control experiment was set up in which hemolysis was produced by lowering the NaCl concentration of the suspending solution (fragility test). Red cells were suspended in 0.4 per cent saline which, it was found, produced a distinct but not complete hemolysis. However, the partly hemolyzed red cells gave the same 4 plus reaction with anti-A, anti-B, and anti-Rh sera as the control. Therefore, the loss of agglutinability by anti-Rh serum is not caused by the process of hemolysis accompanying the heating.

It is known that heated red cells are less agglutinable by weak anti-A and anti-B sera. However, the titers of the used anti-A, anti-B, and anti-Rh sera were the same— $\frac{1}{128}$ ; the titer of the Rh blocking serum was  $\frac{1}{32}$ , and the titer of the anti-N serum was  $\frac{1}{2}$ . Therefore, the differences in the agglutinability between heated and nonheated cells cannot be explained by weakness of the anti-Rh serum.

One may only speculate about the cause of the described observation. Since a similar difference is known to exist between the H and the O agglutinins of some bacilli and since this difference is connected with the location of the agglutinogens, it may be that the A and B and the M and N agglutinogens are situated within the cells, whereas the Rh agglutininogen may be located on the surface.<sup>1</sup> It may be that the Rh agglutinogens are less numerous than the others, as suggested by Wiener,<sup>2</sup> and therefore are destroyed faster. A third possibility is a difference in the chemical structure of the different agglutinogens.

#### SUMMARY

Heating of red cells to 56° C. for five to twenty minutes diminishes or destroys their agglutinability by anti-Rh agglutinating and blocking sera, whereas the agglutinability by anti-A, anti-B, anti-M, and anti-N sera is apparently not affected.

Addition of formalin (0.1 to 1.0 per cent) to red cell suspensions reduces the agglutinability by anti-Rh serum much more than the agglutinability by anti-A or anti-B sera.

While this paper was being prepared for publication, Calvin and associates<sup>3</sup> reported upon a similar investigation in which the same conclusions were arrived at regarding the heat sensitivity of the Rh antigen. They suggest a protein denaturation as a basis for the destruction of the antigen.

#### REFERENCES

1. Wiener, A. S.: Factors Rh and Anti-Rh and Their Applications in Clinical and Legal Medicine, in *The 1941 Yearbook of Pathology and Immunology*, pp. 499-505.
2. Wiener, A. S., Sonn, E. B., and Belkin, R. B.: *The Nature of Rh Agglutination Reactions*, Science 100: 98-99, 1944.
- Wiener, A. S.: Recent Developments in the Knowledge of the Rh-Hr Blood Types; Tests for Rh Sensitization, *Am. J. Clin. Path.* 16: 477-497, 1946.
3. Calvin, M., Evans, R. S., Behrendt, V., and Calvin, G.: Rh Antigen and Hapten; Nature of Antigen and Its Isolation From Erythrocyte Stroma, *Proc. Soc. Exper. Biol. & Med.* 61: 416-419, 1946.

## BENZIDINE-NEGATIVE STOOLS DURING IRON THERAPY

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THE possibility that the administration of iron salts to patients with suspected gastrointestinal bleeding will interfere with the benzidine reaction of the stools is of considerable practical importance. Our interest in this problem was enhanced by the clinical significance of the question, since it is the patient with possible gastrointestinal bleeding who most often is in need of iron therapy. The present study was undertaken in an attempt to clarify this point.

Neither the literature nor laboratory texts offer a definitive answer. Ogilvie<sup>4</sup> believe that iron compounds do not interfere with the benzidine test and suggested that the exclusion of meat, excepting in large quantities, was unnecessary. Johnson and Oliver<sup>3</sup> found false-positive benzidine reactions following the administration of considerable amounts of meat, but the benzidine test was negative on iron administration. In their series seven of the patients received 12 gr. of ferrous sulfate and nine received 30 grains. Bell,<sup>1</sup> on the other hand, found the benzidine test more sensitive than the guaiac test and recommended that iron compounds, as well as iodides, be eliminated from the diet during search for occult blood. Burger<sup>2</sup> concluded, from the two cases studied, that iron and ammonium citrate do not interfere with the guaiac test but that the benzidine test is consistently positive due to the administered iron.

### MATERIAL AND METHODS

Nineteen young women patients, known to be free from gastrointestinal disease, were used in the experiments. In these patients twenty-eight series of observations were made as follows: four during ferrous sulfate administration; seven during ferrous sulfate and copper sulfate administration; six receiving Iberin; one getting Otoferrin; two while on high-iron, meat-free diets; and eight eating high-meat diets.† In most cases meat-free diets were started and two or three successive negative stools were awaited before iron therapy was begun. All patients were kept on the test substance for at least five days, during which time they remained on meat-free diets. Stools were tested before, during, and after the therapy period.

In examining for blood a portion of feces from the center of the specimen was spread out on a piece of white absorbent paper; to this a few drops of freshly prepared benzidine

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†The iron preparations were administered in the following dosage: Ferrous sulfate tablets 0.3 Gm., t.i.d.; Cofron tablets, 2, t.i.d. (solution ferric pyrophosphate, 3.35 gr. copper sulfate, 0.0606 gr. per tablet); Iberin capsules, 3, t.i.d. (iron and ammonium citrate, 5 gr. thiamine hydrochloride, 0.375 mg.; riboflavin, 0.125 mg.; nicotinamide, 5 mg.; liver concentrate, 4 gr. per capsule); Otoferrin, 4 drams, t.i.d. (3.9 gr. of "otoferrin, a form of colloidal iron" [65 mg. iron] per dose).

The diets were approximately the following: The general ward diet contained about 75 to 80 Gm. of protein and 13 or 14 mg. of iron. The high-iron, meat-free diet contained about 90 to 100 Gm. of protein and 20 to 25 mg. of iron. Special attention was given to large servings of whole wheat and oatmeal cereals, eggs, vegetables, (as green peas), fruits (as apricots, peaches, prunes), and bread. The meat-free diet contained about 70 Gm. of protein and 10 to 15 mg. of iron. It was similar to the general ward diet, but milk and eggs were substituted for meat. The high-meat diet contained only 80 Gm. of protein and 14 mg. of iron, but this was derived mostly from meat sources.

TABLE I. SUMMARY OF RESULTS OF STOOL STUDIES ON VARIOUS DIETARY AND THERAPEUTIC REGIMENS									
2	3	4	5	6	7	8	9	10	11

[illegible]

11	Diet Therapy Stool	Meat-free diet 0	→	Cofron 0	→	High-meat diet 0
12	Diet Therapy Stool	Meat-free diet 0	→	Iborin 0	→	Normal diet 0
13	Diet Therapy Stool	Meat-free diet 0	→	Iborin 0	→	High-meat diet 0
14	Diet Therapy Stool	Meat-free diet 0	→	Iborin 0	→	High-meat diet 0
15	Diet Therapy Stool	Meat-free diet 0	→	Iborin 0	→	High-meat diet 0
16	Diet Therapy Stool	Meat-free diet 0	→	Iborin 0	→	High-meat diet 0
17	Diet Therapy Stool	Meat-free diet 0	→	Iborin 0	→	High-meat diet 0
18	Diet Therapy Stool	Meat-free diet 0	→	Iborin 0	→	High-meat diet 0
19	Diet Therapy Stool	Meat-free diet 0	→	Iborin 0	→	High-meat diet 0



solution were applied. The benzidine solution was prepared by adding 5 c.c. of glacial acetic acid to 2 gr. (approximately equivalent to the amount of powdered benzidine on a knife tip) of powdered benzidine in a clean, dry test tube. When this was partially or completely dissolved,  $2\frac{1}{2}$  c.c. of hydrogen peroxide were added. The same reagents were used throughout the determinations, and the benzidine solutions were frequently tested with blood diluted in water and stools known to contain occult blood. The benzidine test was interpreted as follows: plus, thirty seconds or more for the development of a bluish-green reaction; 2 plus, fifteen to thirty seconds for the development of a bluish-green color reaction; 3 plus, five to fifteen seconds; 4 plus, immediate development of the color (less than five seconds).

#### DISCUSSION

Stools from only eight patients were obtained on the first day of a meat-free diet. Of these stools four were positive for blood. One patient had benzidine-positive stools the second and third days as well. The others had benzidine-negative stools after the first day. Five out of eight patients who were placed on high-meat diets developed benzidine-positive stools. In these patients the benzidine test became positive twenty-four to forty-eight hours after the meat was added to the diet and remained intermittently positive during the period of observation.

The eighteen patients who received some form of iron therapy received it over a period of 118 days. During this time eighty-one stool examinations were performed. In this series only one stool (Patient 10) was found to be benzidine positive during iron administration (Table I).

#### SUMMARY AND CONCLUSIONS

1. Eighteen patients on meat-free diets were given various iron preparations in therapeutic doses. The stools, with a single exception, remained negative to the benzidine test.

2. Stools became negative to the benzidine test in a maximum of three days on meat-free diets.

3. The majority of patients tested had benzidine-negative stools even on normal diets.

4. High food-iron, but meat-free diets did not produce stools with positive benzidine reactions.

5. High-meat diets frequently gave positive stool benzidine reactions within twenty-four to forty-eight hours of their initiation.

#### REFERENCES

1. Bell, J. R.: Occult Blood in Faeces, *Guy's Hosp. Rep.* 73: 20, 1923.
2. Burger, G. N.: Influence of Iron on Benzidine and Guaiac Tests for Occult Blood, *Guy's Hosp. Rep.* 84: 112, 1934.
3. Johnson, A. S., and Oliver, E. B.: Effect of Ingested Iron on Tests for Occult Blood in Stools, *J. LAB. & CLIN. MED.* 26: 727, 1941.
4. Ogilvie, A. G.: Testing for Occult Blood in the Faeces; Study of Alleged Fallacies, *Brit. M. J.* 1: 755, 1927.

# HYPERPARATHYROIDISM AND PARTIAL HEART BLOCK

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THE effect of alteration in the level of serum calcium on the heart, as evidenced in the electrocardiogram, has been known for some time. Hypocalcemia leads to a prolonged electrical systole, a prolonged Q-T interval,<sup>1, 2</sup> while hypercalcemia leads to a short Q-T interval.<sup>1, 3</sup>

The effect of serum calcium changes on the P-R interval is less clear. Gold and Edwards<sup>4</sup> injected parathyroid hormone into dogs and found varying degrees of heart block. Others<sup>5</sup> injected calcium chloride into dogs, or man, and found among other things recurring A-V block. Atropine was found to prevent the prolongation of the P-R interval when calcium was given to rabbits,<sup>6</sup> showing that calcium stimulates vagal activity. Furthermore, in guinea pig heart perfusion experiments, Spealman<sup>7</sup> found that the P-R interval lengthened as the calcium ion concentration in the perfusate increased. The Q-T interval was altered (lengthened) only when the calcium concentration decreased.

On the other hand, Segall and White<sup>8</sup> and Berliner<sup>9</sup> reported no prolongation of the P-R interval when calcium was injected into man. However, one patient with hyperparathyroidism (blood calcium of 19.2 mg. per cent) had a P-R interval of 0.23 second which returned to normal after removal of an adenoma.<sup>3</sup>

This case is reported because of the marked degree of heart block which persisted for some time after a parathyroid adenoma was removed and after the serum calcium fell to subnormal levels. The duration of electrical systole, on the other hand, varied with the serum calcium.

## CASE REPORT

R. C., a 17-year-old boy, who had completely recovered from acute nephritis at the age of 12, developed fatigability, low back pain, and three months later aching in the thighs. Later, polyuria, nocturia, and pain on mastication were noted, and ten months after the onset a fracture of the right humerus occurred. This led to x-ray studies, with the discovery of typical osteitis fibrosa cystica and diffuse demineralization of all bones.

The only noteworthy physical findings were the high pulse pressure, with a blood pressure of 154/50 mm. Hg, and the vigorous heart action, with no sign of an aortic valve lesion or cardiac enlargement. Most observers failed to feel a nodule in the area of the lower pole of the right lobe of the thyroid, but this was noted by Dr. E. Goetsch who removed a parathyroid tumor measuring 2.5 by 5 by 2.5 cm. and weighing 14.5 grams.

Laboratory examination of the blood and urine was negative except that the patient had marked calcinuria, 1.2 Gm. per twenty-four hours, after three days on a low calcium diet. The normal output on that regime is less than 0.2 gram. The preoperative serum calcium was 17, phosphorus 1.8, and alkaline phosphatase 32.2 mg. per cent. The electrocardiogram on admission to the hospital revealed a P-R interval of 0.38 second.

The levels of blood calcium, phosphorus, and alkaline phosphatase and the duration of the R-R, P-R, and Q-T intervals are shown in Table I.

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TABLE I. BLOOD CHEMICAL AND ELECTROCARDIOGRAPHIC CHANGES IN A PATIENT WITH A PARATHYROID ADENOMA

	DATE																
	3/8	3/13	3/14	3/15	3/16	3/18	3/19	3/20	3/21	3/22	3/23	3/24	3/25	3/26	3/27	3/28	4/6
Calcium*	17	16.5		Remov-	12.5	10.0	9.9	8.5		7.5	6.9		6.5	6.9		6.9	8.0
Phosphorus*	1.8	2.1		al of	1.6	1.5	1.4	1.7		2.0	2.0		2.4	2.7		2.8	2.2
Phosphatase*	32.2	28.3		ade-	21.9	25.2	31.4	32.3		34.8	42.0		43.6	36.5		37.2	29.8
R-R interval†			.69	noma		.74			.73			.897			.867		.84
P-R interval†			.38			.24			.20			.24			.25		.21
Q-T interval†			.29			.34			.36			.408			.387		.36
*Values as milligrams per cent.																	
†Time in seconds, average of 3.																	

\*Values as milligrams per cent.

†Time in seconds, average of five consecutive readings in lead where waves were best defined, usually Lead II.

Thus it can be seen that as the serum calcium returned to normal on the third postoperative day the P-R interval shortened from 0.38 to 0.24 second. Six days after operation the P-R interval was 0.20 second, while the serum calcium was falling from 8.5 to 7.5 mg. per cent. However, with a subsequent fall in calcium concentration, the P-R interval was maintained at about 0.24 second, until three weeks after operation when it was 0.21 second. At that time the serum calcium concentration was 8.0 mg. per cent.

It is true that at slower cardiac rates the P-R interval may be somewhat lengthened, but the P-R interval changes in this case are much greater in magnitude than those related to heart rate. Furthermore, regardless of rate, the P-R interval should not exceed 0.20 second at rates under 70 per minute.

It seems obvious that the prolonged hypercalcemia had produced changes in the A-V system which reverted slowly to normal in the weeks after operation. The Q-T interval varied as expected, short in relation to heart rate (R-R) with hypercalcemia and prolonged with hypocalcemia.

Parenteral atropine in doses of 0.6 to 1.2 mg. had no effect on the prolonged P-R interval.

#### DISCUSSION

It is generally admitted that the formulas relating normal Q-T intervals and heart rate are not satisfactory, witness the numerous new formulas<sup>10</sup> (Table II). If Hegglin and Holtzmann's formula<sup>11</sup> is used, the patient had a Q-T interval within the normal range preoperatively in spite of marked hypercalcemia. This Q-T interval of 0.29 second would also be just within the range described by White and Mudd.<sup>12</sup> At any rate it is clear that the chief alteration was in the prolonged P-R interval, 0.38 second.

TABLE II. EXPECTED Q-T INTERVALS BASED ON HEART RATE ACCORDING TO VARIOUS FORMULAS

Q-T INTERVALS	DATE			
	3/14	3/18	3/21	3/24
Actual	.29	.34	.36	.41
Expected:				
Schlamowitz <sup>10</sup>	.309	.319	.317	.351
Hegglin and Holtzmann <sup>11</sup>	.324 ± .04	.335 ± .04	.333 ± .04	.369 ± .04
Ashman <sup>14</sup>	.33	.34	.34	.369
Bazett <sup>15</sup>	.308	.32	.32	.35
Frederica <sup>16</sup>	.337	.345	.34	.367

The experimental evidence for A-V block due to hypercalcemia is found in the isolated fiber technique,<sup>13</sup> in which it was shown that increases in calcium concentration eventually produced neuromuscular block. Excess of ionized calcium gradually blocked nerve-muscle transmission by lowering the electrical excitability of the muscle fiber adjacent to the end plate. However, this would not explain the persistence of the prolonged P-R interval while the serum calcium fell to levels producing latent tetany.

The prolonged P-R interval was probably not due to a vagal effect, as atropine in doses sufficient to cause mydriasis and dryness of the mouth did not alter it. This, then, differs from the form of heart block associated with calcium reported by Hoff and associates.<sup>6</sup> The cause of this heart block is unknown.

## SUMMARY

A case of hyperparathyroidism is presented. It shows marked prolongation of the P-R interval and the expected increase in duration of systole with fall in serum calcium.

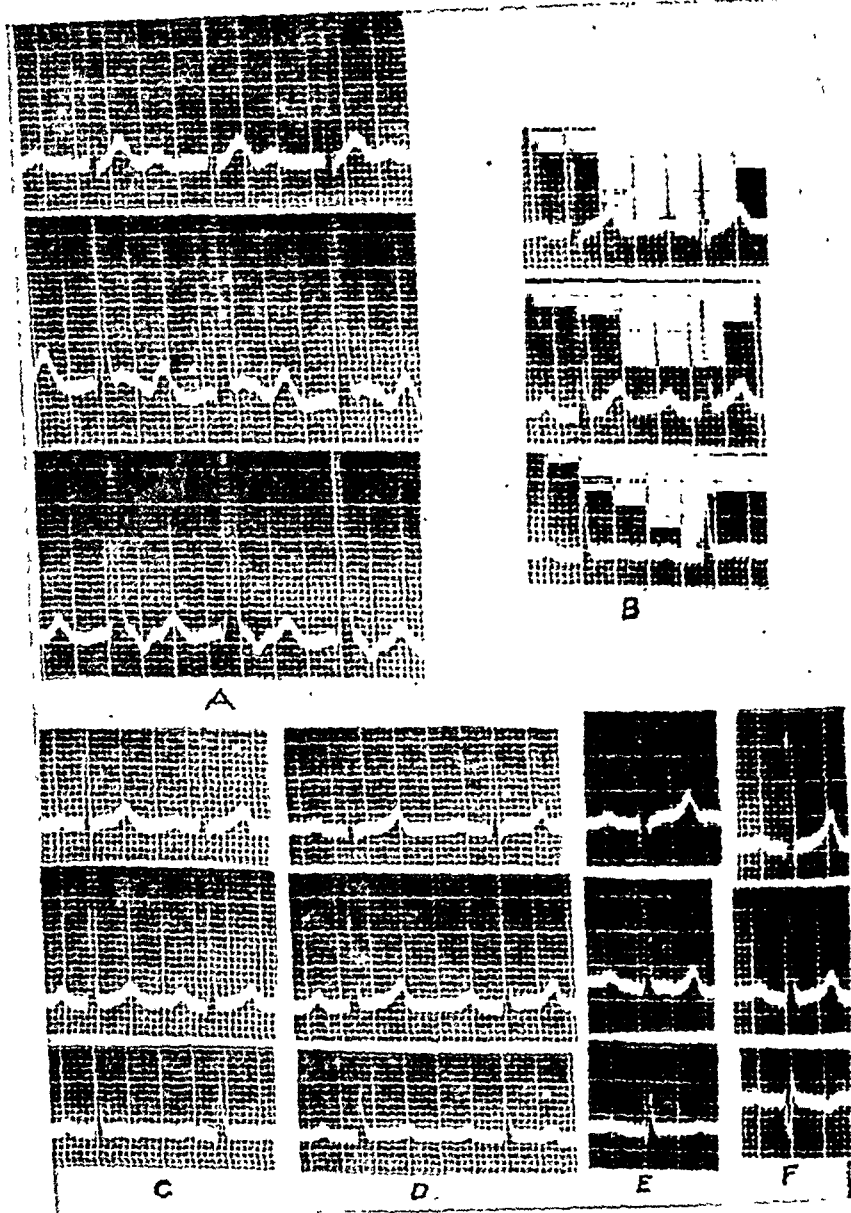


Fig. 1.—Electrocardiograms. Limb leads. (A) Preoperatively. In this film the standardization was inadvertently doubled so that 1 mv. gives a deflection of 2 cm. (B) Postoperatively,  $\frac{1}{2}$ s. (C) Postoperatively,  $\frac{1}{2}$ s. (D) Postoperatively,  $\frac{1}{2}$ s. (E) Postoperatively,  $\frac{1}{2}$ s. (F) Postoperatively,  $\frac{1}{2}$ s.

These two phenomena are independent of each other, as evidenced in Fig. 1D and Fig. 1E, in which a prolonged P-R interval (0.24 second) was found together with abnormally long Q-T intervals, when the serum calcium was below 8 mg. per cent.

Incomplete heart block may be found in the presence of hypercalcemia. It may not completely disappear when the serum calcium level returns to normal or subnormal levels. Atropine did not alter the P-R interval in this case. The effect of changes in blood calcium on the P-R interval is still obscure and needs further study.

## REFERENCES

1. Carter, E. P., and Andrus, E. C.: Q-T Interval in Human Electrocardiogram in Absence of Cardiac Disease, *J. A. M. A.* 78: 1922, 1922.
2. Barker, P. S., Johnston, F. D., and Wilson, F. N.: The Duration of Systole in Hypocalcemia, *Am. Heart J.* 14: 82, 1937.
3. Kellogg, F., and Kerr, W. J.: Electrocardiographic Changes in Hyperparathyroidism, *Am. Heart J.* 12: 346, 1936.
4. Gold, H., and Edwards, D. J.: The Effects of Ouabain on the Heart in the Presence of Hypercalcemia, *Am. Heart J.* 3: 45, 1927.
5. (a) Walters, W., and Bowler, J. P.: Pre-operative Preparation of Patients With Obstructive Jaundice; an Experimental Study of the Toxicity of Intravenous Calcium Chloride Used in the Preparation of Patients, *Surg., Gynec. & Obst.* 39: 200, 1924.  
(b) Fujimori, K.: Clinical Investigation of the Influence of Calcium Upon the Circulatory System; the Influence of Calcium Upon the Electrocardiogram, *Acta scholae med. univ. imp. in Kioto* 16: 36, 1933.
6. (a) Hoff, H. E., and Nahum, L. H.: An Analysis of the Cardiac Irregularities Produced by Calcium, and Their Prevention by Sodium Amytal, *J. Pharmacol. & Exper. Therap.* 60: 425, 1937.  
(b) Winkler, A. W., Hoff, H. E., and Smith, P. K.: Cardiovascular Effects of Potassium, Calcium, Magnesium, and Barium; Experimental Study of Toxicity and Rationale of Use in Therapeutics, *Yale J. Biol. & Med.* 13: 123, 1940.
7. Spealman, C. R.: The Action of Ions on the Mammalian Heart, *Am. J. Physiol.* 136: 332, 1942.
8. Segall, H. N., and White, P. D.: Clinical Observations on the Value of Calcium Chloride as a Diuretic, and Its Influence Upon the Circulatory Mechanism, *Am. J. M. Sc.* 170: 647, 1925.
9. Berliner, K.: The Effect of Calcium Injections on the Human Heart, *Am. J. M. Sc.* 191: 117, 1936.
10. Schlamowitz, I.: An Analysis of the Time Relationships Within the Cardiac Cycle in Electrocardiograms of Normal Men, *Am. Heart J.* 31: 329, 1946.
11. Hegglin, R., and Holzmann, M.: Die klinische Bedeutung der verlängerten QT-Distanz (Systolendauer) im Elektrokardiogramm, *Ztschr. f. klin. Med.* 132: 1, 1937.
12. White, P. D., and Mudd, S. G.: Observations on the Effect of Various Factors on the Duration of Electrical Systole of the Heart as Indicated by the Length of the Q-T Interval of the Electrocardiogram, *J. Clin. Investigation* 7: 387, 1929.
13. Kuffler, S. W.: The Effect of Calcium on the Neuromuscular Junction, *J. Neurophysiol.* 7: 17, 1944.
14. Ashman, R.: The Normal Duration of the Q-T Interval, *Am. Heart J.* 23: 522, 1942.
15. Bazett, H. C.: An Analysis of the Time-Relations of the Electrocardiogram, *Heart* 7: 353, 1920.
16. Frederica, L. S.: Duration of Systole in the Electrocardiogram, *Acta med. Scandinav.* 53: 469, 1920.

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## SENSITIVITY OF *EBERTHELLA TYPHOSA* TO PENICILLIN FRACTIONS G AND X

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VARIOUS investigators have shown the increased effectiveness of penicillin X over other types of penicillin in inhibiting the growth of various organisms in vitro and in exhibiting an increased protective action in vivo.<sup>1,3</sup> This increased activity of penicillin X is not only directed against organisms which are highly sensitive to penicillin but also applies to gram-negative bacilli such as *Klebsiella pneumoniae*<sup>1</sup> and *Escherichia coli*<sup>2</sup> which are relatively resistant. The strain of *Esch. coli* used by Libby and Holmberg<sup>2</sup> was inhibited by 133.9 units of penicillin G and by 46.875 units of penicillin X. Recently, Bigger<sup>4</sup> has shown that *Eberthella typhosa* is inhibited in the test tube by penicillin and that this action of penicillin is potentiated by sulfathiazole. Evans<sup>5</sup> reported that most of the sixty-four strains of *E. typhosa* tested were inhibited by 5 to 10 units of penicillin. Clinical confirmation of Bigger's work was obtained by McSweeney<sup>6</sup> who treated several cases of typhoid fever with a combination of penicillin and sulfathiazole. Relatively large doses of penicillin were used, 10,000,000 units being given over a period of four days, in combination with 34 Gm. of sulfathiazole. In some cases this regimen was repeated, and encouraging results were obtained. While this dose of penicillin appears to be large when expressed in units, actually it is rather small when expressed in grams. Ten million units of penicillin are equal to only 6 Gm., and this was given over a four-day period (1.5 Gm. per day). Actually over five times as much sulfathiazole as penicillin was administered. Owing to the low toxicity of penicillin it can be given in quantities which will result in blood levels of 40 to 80 units per cubic centimeter of serum. It is, therefore, logical to treat diseases in which the infecting organism is susceptible to amounts of penicillin which can be maintained in the blood stream by giving large doses of the drug. Indeed, in view of the results obtained in syphilis by Lloyd-Jones and Allen<sup>7</sup> with a single daily injection of penicillin, there is some question as to the necessity of maintaining high levels over long periods of time. In view of the interest in the treatment of typhoid fever with penicillin, it seemed pertinent to report some results obtained several years ago on the action of penicillins G and X on *E. typhosa*.

Two methods were used for determining sensitivity. The first was a serial dilution method in which dilutions of both of the penicillin fractions and culture under test were made in broth. The inoculum consisted of a 1 per cent dilution of an overnight broth culture of the organism in the test medium. The tests

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were incubated in a water bath at 37° C. and results read at six hours and again at twenty-four hours. The end point was the smallest amount of penicillin resulting in complete inhibition of growth. Twenty-nine strains of *E. typhosa* were studied using the serial dilution technique. The second method was a cylinder plate assay similar to that described for *Staphylococcus aureus*,<sup>8</sup> except that instead of the latter organism the various strains of *E. typhosa* were used as the test organism. Since five strains produced unsatisfactory growth in the seed layer, only twenty-four cultures were tested by this technique. The plate method employs two dosage levels of penicillin, 5 and 20 units (ratio 1 to 4). The penicillin G (370 units per milligram) was considered to be the standard and the penicillin X (900 units per milligram) was treated as the unknown. The potency of penicillin X was computed according to the method of Knudsen and Randall.<sup>8</sup>

The penicillin G was the F.D.A.\* working standard which had a potency of 370 units per milligram when compared to the master standard, crystalline sodium penicillin, which has a potency of 1,667 units per milligram. On the same basis crystalline penicillin X has a potency of 900 units per milligram. These potencies are all based on the *Staph. aureus* plate assay. The F.D.A. working standard had been shown to consist largely of the G fraction.

In Table I are shown the results obtained with twenty-nine typhoid strains in the serial dilution test. At six hours only three strains were inhibited by 0.75 units of penicillin G, while twenty-eight strains failed to grow in the same amount of penicillin X. In twenty-four hours the difference is less striking since twenty-six strains are inhibited by 3 units or less of penicillin X, while twelve strains failed to grow in the presence of the same amount of penicillin G. Of the twenty-nine strains tested by the dilution method, twenty-four were twice as sensitive to penicillin X as to penicillin G on a unit per milligram basis, one strain was three times as sensitive, and four strains were four times as sensitive.

TABLE I. EFFECT OF PENICILLINS X AND G ON *E. TYPHOSA*

		6 HR.				24 HR.			
		<i>Type G Serial Dilution Method</i>							
Number of strains inhibited		3	17	9	2	10	12	4	1
Units per cubic centimeter for complete inhibition		0.75	1.5	3	1.5	3	6	12	>12
		<i>Type X Serial Dilution Method</i>							
Number of strains inhibited		28	1		3	12	11	3	
Units per cubic centimeter for complete inhibition		0.75	1.5		0.75	1.5	3	6	

TABLE II. POTENCY OF PENICILLIN X EXPRESSED AS UNITS PER MILLIGRAM AND PER CENT OF STANDARD (G)

Number of strains showing increased potency	1	5	9	1	2	3
Potencies of "X" in units per milligram; G equals 1,667 u./mg.	1800 to 2700	2700 to 3600	3600 to 4500	4500 to 5400	5400 to 6300	6300 to 7200
Potencies of "X" expressed as per cent of standard (G)	200 to 300	300 to 400	400 to 500	500 to 600	600 to 700	700 to 800

\*F.D.A., Food and Drug Administration.



In Table II the results of the cylinder plate tests are given. This method of testing reveals an even greater difference between the two penicillin species than was shown by the serial dilution test. All strains were at least twice as sensitive to penicillin X as to penicillin G, and three strains were seven or eight times as susceptible. If the potency of penicillin X is computed in units per milligram, the activity varies from 1,800 units per milligram to 7,200 units per milligram, depending on the strain of *E. typhosa* used for assay.

#### DISCUSSION

The known properties of penicillin and streptomycin indicate that the latter drug should be the one of choice for the treatment of gram-negative infections. Yet recent experience with streptomycin in the treatment of typhoid fever<sup>9</sup> indicates that this antibiotic is of questionable value in spite of apparent in vitro sensitivity of the typhoid organism to it. Conversely, although it is generally accepted that the typhoid organism is resistant to penicillin, McSweeney<sup>6</sup> has successfully treated cases with this drug augmented by sulfathiazole. As a matter of fact, based on the data presented in these studies and those of Evans,<sup>5</sup> there is reason to believe that penicillin alone should be an effective treatment in this disease when given in the proper doses. On the basis of these studies all twenty-nine strains of *E. typhosa* were inhibited in a yeast-beef broth at pH 7.0 by approximately 12 units per cubic centimeter or less of penicillin (7.2 gamma). When two of these strains were tested in the same broth for their sensitivity to streptomycin, 25 gamma were required to effect complete inhibition. Thus, on a weight basis, over three times as much streptomycin as penicillin was required for inhibition of these strains. When a comparison of the resistance of *E. typhosa* to the two antibiotics is made on a weight basis, the apparent advantage of streptomycin because of its characteristic attack on the gram-negative organisms is considerably minimized. Furthermore, the characteristic rapid and marked increase in resistance of gram-negative organisms to streptomycin both in vivo<sup>10</sup> and in vitro<sup>11</sup> argues against the therapeutic use of this drug, particularly where another less toxic drug (penicillin) is available without these disadvantages.

The dose of penicillin which would apparently be effective in the treatment of typhoid fever is not a large one in terms of weight of drug utilized. In Keefer's<sup>9</sup> recent publication on the use of streptomycin in typhoid fever, doses of 3 to 5 Gm. per day are recommended. Doses of penicillin such as these should result in blood levels more than adequate to be effective against the most resistant strains of *E. typhosa* reported in these studies or in those reported by Evans.<sup>5</sup> The wide range between the therapeutic and toxic doses of penicillin as compared to the range between these doses of streptomycin is further argument in favor of penicillin therapy for typhoid fever.

Unfortunately, at the present time there is no commercial production of penicillin X. There is little advantage, if any, to the production of this penicillin fraction at the expense of the regular production of commercial penicillin (largely fraction G). So far, penicillin X has been shown to be quantitatively superior to penicillin G only; until a qualitative difference between penicillins X

and G can be demonstrated which would warrant the extra expense of producing penicillin X, it is unlikely that it will become commercially available. It is obvious, however, that in the case of typhoid fever considerably less than half as much penicillin X as penicillin G would be necessary in the treatment of this disease.

#### SUMMARY AND CONCLUSIONS

1. Of twenty-nine strains of *E. typhosa* tested all except one were inhibited by 12 units or less of penicillin G, in twenty-four hours.
2. All of the strains were inhibited by 6 units or less of penicillin X.
3. When the plate method was used to determine sensitivity, penicillin X was from two to eight times as effective as penicillin G.
4. A favorable response should be obtained in the treatment of typhoid fever provided sufficiently large doses of penicillin are utilized.

#### REFERENCES

1. Welch, H., Putnam, L. E., Randall, W. A., and Herwick, R. P.: Penicillin X; Successful Treatment of Gonorrhea With a Single Intramuscular Injection, *J. A. M. A.* 126: 1024, 1944.
2. Libby, R. L., and Holmberg, N. L.: The Activity of Penicillin G and X in Vitro, *Science* 102: 303, 1945.
3. Hewitt, W. L.: Antibacterial Action of Penicillin, Penicillin X, and Streptomycin on *Hemophilus Influenzae*, *Pub. Health Rep.* 61: 768, 1946.
4. Bigger, J. W.: Synergistic Action of Penicillin and Sulfathiazole, *Lancet* 250: 81, 1946.
5. Evans, R. W.: Penicillin Sensitivity of *Bact. Typhosum*, *Lancet* 251: 113, 1946.
6. McSweeney, C. J.: Sulphathiazole and Penicillin in Typhoid Fever, *Lancet* 251: 115, 1946.
7. Lloyd-Jones, T. R., and Allen, S. J.: Out Patient Treatment of Early Syphilis With Penicillin, *Brit. M. J.* 567: 1946.
8. Knudsen, L. F., and Randall, W. A.: Penicillin Assay and Its Control Chart Analysis, *J. Bact.* 50: 187, 1945.
9. Keefer, C. S.: Streptomycin in the Treatment of Infections, *J. A. M. A.* 132: 70, 1946.
10. Finland, M., Murray R., Harris, H. W., Lawrence, K., and Meads, M.: Development of Streptomycin Resistance During Treatment, *J. A. M. A.* 132: 19, 1946.
11. Miller, C. P., Bohnhoff, M.: Streptomycin Resistance of Gonococci and Meningococci, *J. A. M. A.* 130: 485, 1946.

# PHOSPHATASES AND INORGANIC PHOSPHORUS IN NORMAL HUMAN SERUM

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INVESTIGATIONS of a clinical nature involving a consideration of the levels of human serum phosphatases and inorganic phosphorus necessitated the establishment of normal values in men and women. A group of 101 adults and 108 school children, aged 10 to 12 years, from the country around Edmonton volunteered to give blood in the fall of 1945, and their sera were assayed. According to Klasmer,<sup>1</sup> who worked with infants, the normal values for children have not been established with accuracy, and it was felt that the previously mentioned age group might be of interest. A seasonal variation in phosphatase activity was noted by Klasmer, and in order to find the effect of winter in the Edmonton area, estimations of inorganic phosphorus, acid, and alkaline phosphatases were repeated in the spring of 1946 with the sera of as many as possible of the fall donors.

## EXPERIMENTAL

Serum phosphorus and serum phosphatases were determined by the micro-method of Shinowara and associates<sup>2</sup> as modified by Gould and Schwachman.<sup>3</sup> Acid phosphatase was estimated at pH 5.3 and alkaline phosphatase at pH 9.3.

The unit of phosphatase activity is defined by Shinowara and associates as equivalent to 1 mg. of phosphorus as phosphate ion liberated during one hour of incubation at 37° C., with a substrate containing sodium  $\beta$ -glycerophosphate, hydrolysis not exceeding 10 per cent of the substrate, and optimum pH of the reaction mixture for "acid,"  $5.00 \pm 0.15$ , and for "alkaline,"  $9.30 \pm 0.15$ . Our estimations of phosphatase activity comply with this definition, except with regard to the optimum pH for acid phosphatase. The pH optimum is, however, a broad one, and we found no significant variation when estimations were made at pH 5.0 or 5.3.

## RESULTS AND DISCUSSION

The mean values and the range of values for the different groups are summarized in Table I.

The acid phosphatase values which are very close to the range of 0.0 to 1.1 units reported by Shinowara show an increase during the winter in the adult groups. The Gutman and Gutman<sup>4</sup> range is 0.5 to 2.0 units.

Important variations are found in alkaline phosphatase levels in relation to sex, age groups, and season. For both children and adults the values for male subjects are appreciably higher than for female subjects (30 and 20 per cent, respectively). The activity of the alkaline enzyme is approximately three

TABLE I. NORMAL MEAN VALUES (RANGE GIVEN IN BRACKETS) FOR HUMAN SERUM PHOSPHATASES AND INORGANIC PHOSPHORUS IN RELATION TO SEX, SEASON, AND AGE GROUP

BLOOD DONORS	NUMBER OF SERA	ACID PHOSPHATASE (UNITS PER 100 C.C. SERUM)	ALKALINE PHOSPHATASE (UNITS PER 100 C.C. SERUM)	SERUM INORGANIC PHOSPHORUS (MG./100 C.C.)
<i>Adults</i>				
Men				
Fall	38	0.38 (0.0 to 1.2)	4.4 (2.0 to 7.0)	4.6 (2.6 to 5.4)
Spring	14	0.44 (0.0 to 1.2)	4.8 (2.0 to 7.3)	4.4 (3.2 to 5.4)
Women				
Fall	63	0.37 (0.0 to 1.2)	3.8 (2.0 to 7.0)	4.4 (3.2 to 5.4)
Spring	42	0.67 (0.0 to 1.2)	4.1 (2.5 to 7.3)	4.0 (1.8 to 5.4)
<i>Children, 10 to 12 Years</i>				
Boys				
Fall	51	—	12.8 (6.0 to 21.8)	5.9 (4.6 to 7.8)
Spring	44	0.61 (0.0 to 1.6)	13.5 (5.6 to 21.4)	4.9 (3.0 to 7.8)
Girls				
Fall	57	—	9.4 (6.0 to 17.8)	6.0 (4.8 to 7.8)
Spring	46	0.56 (0.0 to 1.6)	12.1 (5.6 to 17.1)	5.2 (2.0 to 6.4)

times greater among children than adults for both sexes. Bodansky<sup>5</sup> reports a range of 1.5 to 4.0 units for adults and of 5 to 12 units for children.

The phosphorus values follow a similar though less marked trend. There is a very definite seasonal variation in the levels of phosphorus and alkaline phosphatase. In all groups there is a lower amount of serum phosphorus by spring, more especially in the case of children. At the same time, the winter has produced a definite rise in the amounts of alkaline phosphatase. Although active rickets was not observed in our donors the results noted corroborate Klasmer's suggestion that the seasonal variations noted may be associated with a winter diet and decreased exposure to ultraviolet rays.

#### SUMMARY

Serum phosphatases and inorganic phosphorus values were determined in the fall of 1945 on 101 adults and 108 children, aged 10 to 12 years. The estimations were repeated in the spring with fifty-six of the adults and ninety of the children. The average fall level of 11.1 Shinowara units for serum alkaline phosphatase for the children was almost three times that for the adults of 4.1 units. The activity of this enzyme was 20 to 30 per cent greater among the male than among the female subjects. An increase in the alkaline enzyme and a decrease in inorganic phosphorus, which were more marked in children than in adults, may be due to a winter diet and lack of sunshine. An increase in acid phosphatase was noted in the spring values of the adult groups.

#### REFERENCES

1. Klasmer, R.: Serum Phosphatase Activity and Clinical Rickets in Children in Jerusalem, *Am. J. Dis. Child.* 67: 348, 1944.
2. Shinowara, G. Y., Jones, L. M., and Reinhart, H. L.: The Estimation of Serum Inorganic Phosphate and "Acid" and "Alkaline" Phosphatase Activity, *J. Biol. Chem.* 142: 921, 1942.
3. Gould, B. S., and Schwachman, H.: A New Method for the Bio assay of Antiscorbutic Substances, *J. Biol. Chem.* 151: 439, 1943.
4. Gutman, L. B., and Gutman, A. B.: Estimation of "Acid" Phosphatase Activity of Blood Serum, *J. Biol. Chem.* 136: 201, 1940.
5. Bodansky, A.: Phosphatase Studies. II. Determination of Serum Phosphatase. Factors Influencing the Accuracy of the Determination, *J. Biol. Chem.* 101: 93, 1933.

# LABORATORY METHODS

## RAPID CONTINUOUS EXTRACTION AND DETERMINATION OF QUININE

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THE urgency of antimalarial work during the past few years has emphasized the importance of further studies on quinine as well as the search for new drugs. The ultimate progress of such studies is determined by the quantitative methods which are applicable to biologic material. Many improved methods have been reported recently by various workers.<sup>1-9</sup> Each of these methods requires the preliminary separation of the alkaloid from the biologic sample. In most of these this separation is accomplished by extraction with a suitable solvent. Ether,<sup>3</sup> alcohol and ether,<sup>4</sup> acetone and ether,<sup>9</sup> chloroform,<sup>1,2</sup> and ethylene dichloride<sup>7</sup> are proposed either for stepwise manual extraction in a separatory funnel<sup>2, 4, 7, 9</sup> or for a continuous process in specially designed apparatus.<sup>1, 3</sup> One method, designed for blood plasma, employs a filtrate which retains the quinine after the proteins are precipitated with metaphosphoric acid.<sup>5</sup> Once the alkaloid is separated from the sample, its measurement is based on either turbidimetric,<sup>3, 8</sup> fluorometric,<sup>1, 4</sup> colorimetric,<sup>2, 7, 9</sup> or spectrophotometric<sup>6, 10</sup> instrumentation.

Each of these methods offers advantages depending upon the material which is available and the requirements which are imposed by a particular investigation. Recently, certain investigators<sup>11, 12</sup> have applied two or more methods simultaneously to the same materials. This has enhanced the value of the data and enabled additional interpretations, because no method which is specific for quinine<sup>8</sup> has been designed and each when applied biologically may yield results which are vitiated by an indefinable amount of metabolic products.

In this report modifications of certain previous methods for quinine are described and adapted to a procedure which reduces the time and work of extraction and provides an extract of a biologic sample to which duplicate methods of assay may be applied simultaneously. These modifications became urgent during a study of the absorption of quinine in which the analysis of numerous fresh samples was required within brief periods. The existing methods which require manual manipulation of individual samples throughout the extraction become laborious for the analyst and limit his capacity. Also, the extended time which is required for continuous extraction by present methods<sup>1, 3</sup> impedes concentrated periods of work. The time required for the continuous extraction of quinine from a sample of tissue has been reduced from four hours to fifteen minutes. This has been accomplished by changing

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the design of the sample tube of an extractor previously recommended for continuous use with ether,<sup>3</sup> so that it accommodates the continuous flow of a heavier-than-water solvent such as chloroform. The extractor permits a greater flow of solvent through the sample than its predecessor. Also, the solubility of quinine is higher in chloroform than in ether. Both of these factors contribute to the more rapid extraction.

#### APPARATUS

The extractor is similar in principle to others which have been reported<sup>13-15</sup> for continuous liquid-liquid extraction with a solvent of greater density than water. Additional features are described which render the apparatus efficient and convenient for the biologic study of quinine and other alkaloids. It is simple to construct and is an interchangeable part of a unit for continuous operation with a solvent of less density<sup>3</sup> which has had extended use in this laboratory. The adaptability of the complete unit to solvents of greater or less density than water expedites the analysis of identical samples by different methods as previously recommended. The details of construction and the interrelation of parts shown in Fig. 1 are self-explanatory. Additional versatility of the unit derives from its construction in different sizes, among which certain parts are interchangeable. The dimensions are presented in Table I and are indicated according to the labels in Fig. 1.

TABLE I. SPECIFICATIONS FOR CONSTRUCTION OF EXTRACTORS  
(ALL DIMENSIONS IN MILLIMETERS)

REFERENCE TO FIG. 1		VOLUME OF SAMPLE TO BE EXTRACTED					
UNIT	NUMBER	5 C.C.	10 C.C.	25 C.C.		50 C.C.	100 C.C.
				(A)	(B)		
A	1		22	22		38	38
	2		16	16		32	32
	3		22	22		38	38
	4		16	16		32	32
	5		5	5		8	8
	6		85	85		55	85
	7		85	135		85	135
B	8		200	235		190	240
	9	16	16		32	32	
	10	22	22		38	38	
	11	12	16		24	32	
	12	60	60		60	60	
	13	100	100		100	100	
	14	180	180		180	180	
	15	18	18		20	20	
	16	22	22		38	38	
	17	5	5		8	8	
C	18	32	32	32	52	52	52
	19	140	140	140	140	140	140
	20	80	80	80	80	80	80
	21	325	325	325	325	325	325

The absence of any horizontally connected parts or side arms contributes to a minimum of breakage and enables the compact alignment of multiple units in a battery. Batteries of six units each have proved convenient when mounted on horizontal rods 2 feet in length which are clamped at either end to vertical iron supports. When chloroform is used, smooth operation at the

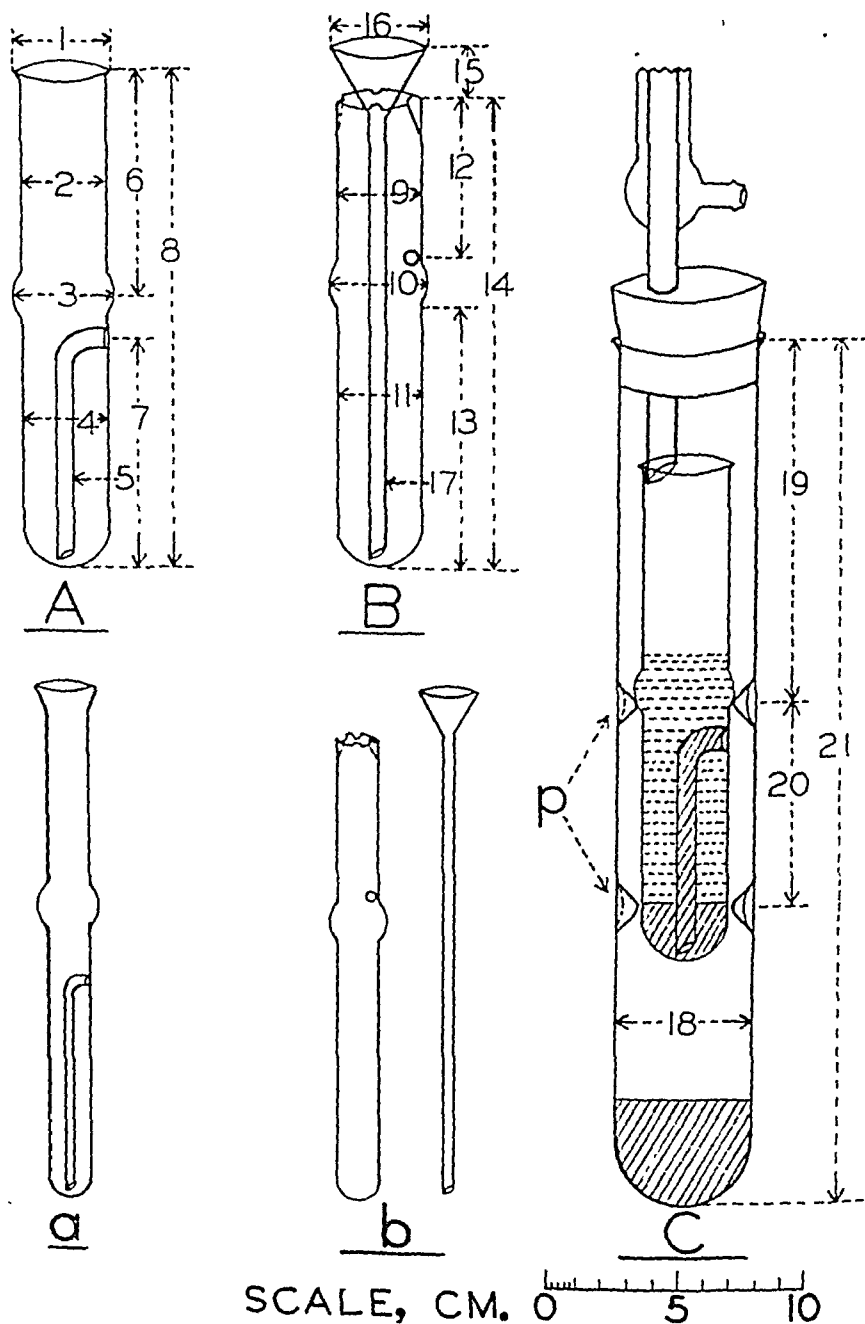


Fig. 1.—Extractors for solvent of greater density: A, 50 c.c.; a, 10 c.c. Extractors for solvent of less density: B, 50 c.c.; b, 10 c.c. Punch marks for support of unit A: p. Complete apparatus, assembled with unit A: C.

beginning of the continuous process demands a readily adjustable source of heat. In this case the units in each battery are heated with a manifold of six ordinary gas burners which are equipped with adjustable chimneys for steady flames. On the other hand, when ether is used, a constant and moderate-rate of heating is required. An electric heater which is easy to construct and has given years of heavy service is recommended.

The heating unit consists of a coil made by winding 140 turns of No. 22 Chromel wire about a  $\frac{3}{8}$  inch mandrel and mounting in a suitable housing. The housing is an elongated right angle trough made by joining two pieces of  $\frac{1}{4}$  inch Transite. Each wall of the trough is 2 by 24 inches on the inside. A support, 3 by 3 inches, is attached at either end. The pieces of Transite are joined securely with small 45 degree brackets and bolts. Small brass bolts through the end supports serve as binding posts for the electrical circuit. A cover,  $2\frac{1}{4}$  by 24 inches, is undercut at 45 degrees along the sides and is provided with six holes, 1 inch in diameter, 4 inches apart, and 2 inches from either end. The unattached cover rests firmly and horizontally. Each extractor unit in a battery rests on its respective hole in the cover of the heater.

Rheostatic control is provided by a resistance whose specifications are identical to those of the heating element. This resistance is mounted with binding posts at one-tenth intervals on the back of a strip of Transite, 3 by 24 inches, which is clamped at each end to the vertical supports for the battery of extractors. Electrical connections are made with the binding posts from the front of the strip, on the back of which the resistance is mounted.

#### PROCEDURE

*Digestion of Tissue.*—Samples of tissue are digested by a procedure which is essentially that of Kelsey and Geiling.<sup>4</sup> Slight modifications are introduced which improve the results after extraction of the alkaloid. The weight of the sample is roughly estimated in advance and defined as 1 part. To a beaker whose capacity is approximately 20 parts is added 5 parts of 2 per cent sodium hydroxide. The gross weight of the beaker, contents, and cover is recorded. Immediately after removal from its source, the sample of tissue is cut into 0.5 to 1.0 Gm. pieces and placed in the alkali. The weight of the sample is established by difference to an accuracy of 1 in 500. Additional alkali to make a total of 10 parts is added, in which the tissue is digested on a steam plate near the boiling point of the alkali. Hot water is added at intervals to compensate for evaporation, so that reduction in volume never exceeds one-half. The alkaline digest is transferred to a volumetric flask, the capacity of which is approximately 20 parts, and diluted to the mark. Aliquots of the resulting 5 per cent solution of tissue in 1 per cent alkali are extracted and analyzed according to the following procedure.

*Extraction of Alkaline Digest.*—An extractor of appropriate dimensions is selected and assembled as in Fig. 1C. Chloroform is added to the inner tube of the unit. The amount of solvent is 25 c.c. for either the 10 or 25 c.c. extractor and 50 c.c. for either the 50 or 100 c.c. extractor. An aliquot of the alkaline digest is introduced by pipette into the inner tube. Solvent which is displaced



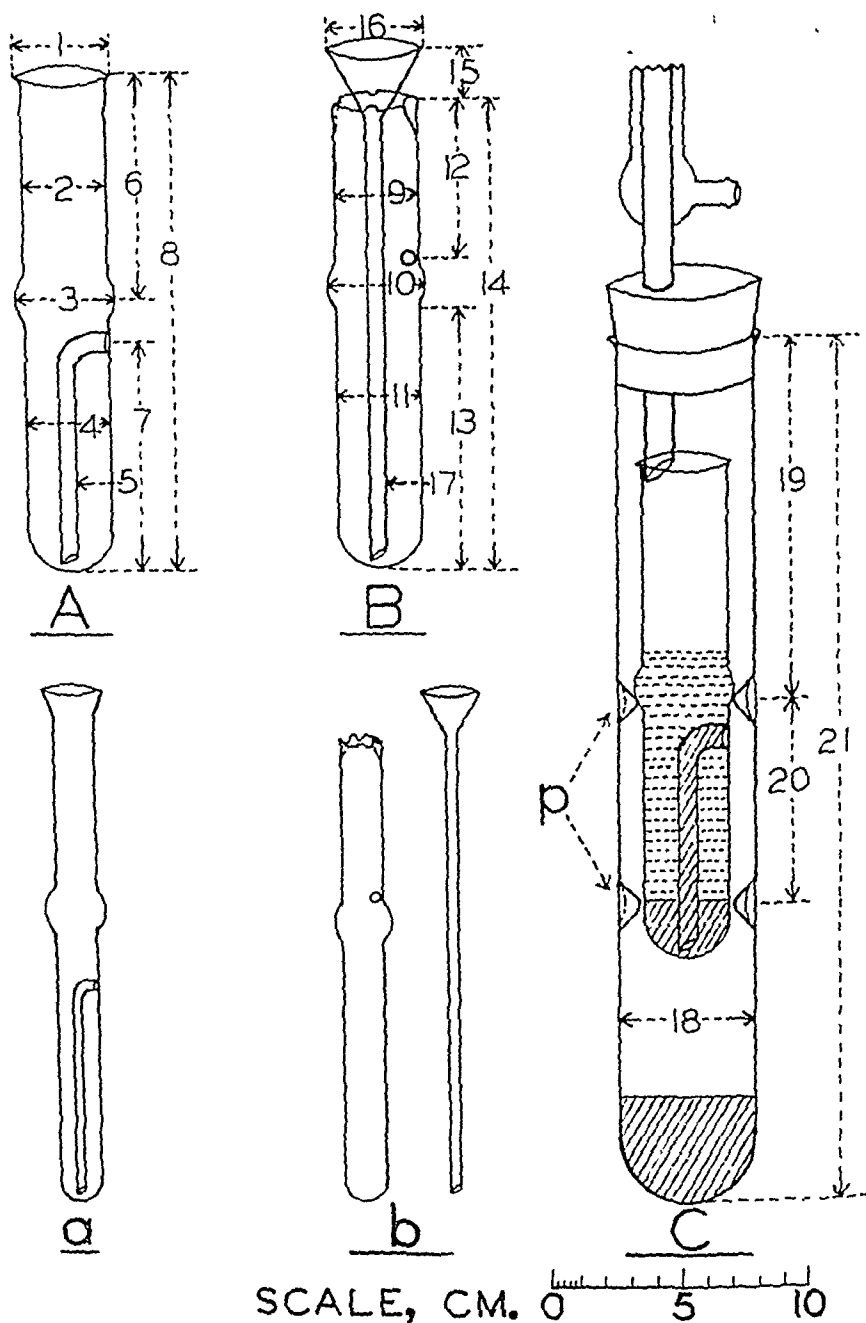


Fig. 1.—Extractors for solvent of greater density: *A*, 50 c.c.; *a*, 10 c.c. Extractor for solvent of less density: *B*, 50 c.c.; *b*, 10 c.c. Punch marks for support of unit *A*: *p*. Complete apparatus, assembled with unit *A*: *C*.

beginning of the continuous process demands a readily adjustable source of heat. In this case the units in each battery are heated with a manifold of six ordinary gas burners which are equipped with adjustable chimneys for steady flames. On the other hand, when ether is used, a constant and moderate rate of heating is required. An electric heater which is easy to construct and has given years of heavy service is recommended.

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by the aliquot overflows into the jacket. The column of solvent which remains underneath the aqueous layer is approximately 1 inch deep. The reflux condenser is attached so that its tip touches lightly the funnel of the extractor. The solvent in the jacket is heated rapidly with a coneless but nonluminous flame until returning solvent begins to drain from the extractor. The flame is then regulated so that refluxing continues at a rate which is consistent with a stable interface at the junction of the solvent and sample. Properly digested samples permit the return of solvent at a rate which overflows in a continuous stream. After brief experience with the apparatus, the initial period of heating and regulation of the continuous rate can be accomplished in one or two minutes. The extraction is continued at the steady rate for 15, 15, 30, and 30 minutes for the 10, 25, 50, and 100 c.c. units, respectively.

*Transfer of Alkaloid to Acid.*—After the extraction is completed, the sample tube is removed from the jacket and set aside. A small funnel is inverted and lowered into the chloroform until it rests on the bottom of the jacket. The stem of the funnel should extend about 2 cm. above the surface of the solvent. A measured quantity of acid is added to the chloroform layer. Either 0.03 N hydrochloric acid, or 0.03 or 0.1 N sulfuric acid, is used, depending on the subsequent procedure which is selected for the analysis. The volume of acid generally equals that of the extracted aliquot, but ratios greater or less than unity are employed when the expected content of the sample indicates any analytic advantage. The condenser is attached again to the jacket and the contents are heated for ten minutes, during which time the funnel actively percolates the chloroform layer through the supernatant acid layer. The condenser is allowed to drain, and any clinging drops on its tip are recovered by tipping against the inner wall of the jacket. The jacket is stoppered tightly with a cork and set aside to cool until the layers are clear and a sharp interface obtains. Aliquots from the acid layer are taken for analysis either by pipetting directly from above the chloroform layer or after separation of the two layers. When pipetting the samples directly, a small mirror is adjusted at 45 degrees in front of the jacket so that the interface is visible and not disturbed by the tip of the pipette.

*Determination of Quinine.*—The quinine in the acid layer is determined by one or more previously established methods which apply directly to a solution of the alkaloid in either hydrochloric or sulfuric acid. When 0.03 N hydrochloric acid is used in the transfer previously described, the method of Kyker and associates<sup>3</sup> is convenient. When the quinine is taken up in 0.03 N sulfuric acid, two methods are directly applicable to the same solution. Either a turbidity of quinine silicotungstate is prepared as previously described<sup>3</sup> for hydrochloric acid solutions and measured by an established calibration with an Evelyn photoelectric colorimeter (Table II), or the fluorescence of quinine sulfate, after dilution with 0.1 N sulfuric acid to a suitable concentration, is read against a similar standard solution with a Coleman photofluorometer. For samples of tissue of very low alkaloidal content, 0.1 N sulfuric acid is used in the preceding transfer from chloroform, and the fluorescence of the alkaloid is measured without further dilution.

TABLE II. MEASUREMENT OF QUININE SILICOTUNGSTATE IN 0.03 N SULFURIC ACID

NUMBER OF TRIPPLICATE SAMPLES ANALYZED	EVELYN COLORIMETER READING		QUININE (MG. PER LITER)		
			ACTUAL	OBSERVED	
	RANGE	AVERAGE		RANGE	AVERAGE
6	90.5 to 91.4	91.0	1.0	1.02 to 1.04	1.01
9	78.2 to 79.9	79.3	2.0	1.92 to 2.06	1.98
9	68.6 to 69.8	69.3	3.0	2.93 to 3.06	2.99
9	60.7 to 62.7	61.8	4.0	3.81 to 4.07	3.96
9	54.2 to 56.1	55.2	5.0	4.81 to 5.14	4.96

The results are calculated as follows:

$C = c \times F \times V \times 1000/W$  where

C = mg. quinine per kg. of tissue

c = mg. quinine per liter in the undiluted acid layer

F = factor of dilution,  $\frac{\text{total acid layer (c.c.)}}{\text{aliquot of alkaline digest (c.c.)}}$

V = total volume of alkaline digest in liters

W = sample of tissue in grams

#### STANDARDIZATION

The procedure which is outlined has been verified by repeated extraction and determination of quinine in standard solutions and in samples of urine and tissue from the alimentary tract of the rat. A summary of these data is presented in Table III. A wide variation in the amount of added quinine is covered, the largest being over 3,000 times the smallest. To the biologic

TABLE III. RECOVERY OF QUININE BY CONTINUOUS EXTRACTION WITH CHLOROFORM

NUMBER OF DETERMI- NATIONS	ANALYSIS		QUININE		
			ADDED MG.	RECOVERED	
	SAMPLE	METHOD*		AVERAGE (%)	RANGE (%)
4	Standard	F	0.005	99.9	$\pm 1.6$
2	Standard	F	0.010	101.6	$\pm 0.0$
4	Standard	F	0.025	96.5	$\pm 1.5$
2	Urine	F	0.025	105.0	$\pm 0.8$
2	Stomach	F	0.025	96.7	$\pm 0.3$
2	Intestine	F	0.025	100.3	$\pm 0.7$
2	Standard	TS	0.050	98.0	$\pm 0.7$
4	Standard	F	0.100	99.2	$\pm 1.2$
2	Standard	TS	0.100	95.5	$\pm 0.5$
2	Stomach	TS	0.100	99.3	$\pm 0.8$
2	Intestine	TS	0.100	98.5	$\pm 0.0$
5	Standard	TS	0.135	99.6	$\pm 1.0$
4	Standard	TS	0.150	99.6	$\pm 2.2$
4	Standard	F	0.250	98.7	$\pm 0.3$
2	Urine	TS	0.250	98.0	$\pm 0.0$
2	Stomach	TS	0.250	98.8	$\pm 0.1$
2	Intestine	TS	0.250	99.4	$\pm 0.4$
4	Standard	F	0.500	100.6	$\pm 0.2$
2	Urine	TS	0.500	97.0	$\pm 0.0$
4	Standard	TS	0.804	98.6	$\pm 1.9$
2	Stomach	TS	1.000	97.2	$\pm 2.1$
4	Intestine	TS	1.000	97.6	$\pm 0.8$
2	Urine	TS	2.500	102.6	$\pm 1.5$
4	Standard	TS	5.000	101.1	$\pm 0.6$
2	Urine	TS	5.000	97.0	$\pm 1.5$
2	Urine	TS	10.000	98.0	$\pm 0.0$
2	Standard	TS	16.075	102.2	$\pm 0.5$

\*F, Fluorometric analysis; TS, turbidimetric analysis of silicotungstate.

samples the calculated volume of a concentrated standard solution of quinine dihydrochloride was added by pipette before digestion. The added amounts of quinine which are recorded in Table III pertain to 25 c.c. aliquots which were extracted. Results from larger or smaller samples with extractors of corresponding dimensions are similar in accuracy.

When standard solutions were analyzed, the preliminary digestion of the sample was omitted and aliquots were extracted after adding 50 per cent sodium hydroxide until a concentration of 1 per cent was reached. For urine samples, digestion with 2 per cent alkali for thirty minutes greatly reduced a tendency to emulsify during the extraction. For samples of tissue, the digestion was continued twenty-four hours.

The accuracy of the procedure is best judged by the results in the last column of Table III. This column represents the duplicability which obtains for similar aliquots. The accuracy of the average recoveries which are found in the preceding column is quite satisfactory for biologic studies of quinine. This column entails any errors in the addition of widely varying amounts of alkaloid, as well as those errors which are inherent for the procedure. The average of all recoveries which are listed in Table III is  $99.1 \pm 0.79$  per cent.

#### APPLICATION AND DISCUSSION

The procedure is especially versatile and efficient in studies where wide variations in the alkaloidal content of the sample occur, such as in a study of the intestinal absorption of quinine at increasing intervals after oral administration. In this connection the method has been used extensively in studies on the rat. The widely used technique of Cori<sup>16</sup> for studying intestinal absorption in small animals does not serve for quinine, since its low solubility at the normal pH of intestinal contents and its tendency to associate with proteins cause it to adsorb on the mucous lining so that the residue cannot be washed out quantitatively. The general application of the method is supported by the work of Alogdelis<sup>17</sup> who used the procedure recently in determining the quinine which remains in the whole body of mice at intervals after oral administration of the drug.

Digestion of a sample of tissue for twenty-four hours may seem a cumbersome step but is recommended on the basis of controlled experiments. Digestion for two hours, as recommended by Kelsey and Geiling,<sup>4</sup> gave extracts which contained appreciable interfering substances. These substances decreased as the digestion was increased. Slight interference remained after eight hours of digestion. Satisfactory blanks were obtained after twenty-four hours. This specified period is longer than is necessary for some tissues but represents more convenience than periods between eight and twenty-four hours, since the digestion of a series of samples may be begun at a given time on one day and their extraction and analysis at the same time on the following day. No attention is required during the digestion except for the occasional replacing of water which evaporates. If flasks, closed with rubber stoppers which support 10 inch air condensers, are preferred to beakers covered with

watch glasses, very little loss of water occurs. Samples are rendered completely soluble except for fragments of bones and hair which remain when the whole carcass of a small animal is digested for analysis.

During the time in which the procedure was developed, chloroform was not readily available and various grades were used. Irregular periods of good and poor results became correlated with the kind of solvent which was in use. It has been substantiated repeatedly that quantitative recoveries of quinine depend on the use of pure chloroform. Merck's reagent and Baker's CP chloroform yield reliable results. The solvents should be stored in amber bottles in a dark cabinet when not in use. Incomplete and irregular recoveries ranging from 75 to 90 per cent occur when U. S. P. or Technical grades of chloroform from various sources are used. The poor recoveries are more noticeable when the content of the sample is low but persist to some extent at higher concentrations. Some poor grades of solvent did improve but were not entirely satisfactory when fractionated or when washed successively with alkali, acid, and water. Used portions of acceptable solvents remained satisfactory when collected in a storage bottle, washed, dried, and distilled before using again.

In the construction of the apparatus (Fig. 1A) it is essential that the tip of the overflow tube inside the extractor is not fused to the bottom of the latter. Otherwise, pumping of the solvent occurs by forming bubbles of vapor in the overflow tube and the aqueous layer may spill over. Without exception, pumping of the chloroform layer and loss of the aqueous layer has occurred only in those extractors in which this oversight of construction existed. If 1 or 2 mm. intervene the tip of the overflow tube and the bottom of the extractor, the returning solvent from the reflux cools the solvent sufficiently to prevent any boiling inside the extractor due to heating by the rising vapors on the outside.

The maximum volume of an aliquot which is to be used in an extractor of particular dimensions is indicated in Table I. This maximum volume is recommended only for relatively pure aqueous solutions of quinine. The products of digestion of tissue with alkali tend to emulsify slightly at the interface during rapid extraction, so that the sample should not exceed 75 to 80 per cent of the rated capacity of the extractor. No difference is noted in recoveries when as low as 50 per cent of the rated volume of sample is used, since the shorter aqueous layer compensates for the slower turnover in the deeper layer of solvent.

The transfer of the extracted alkaloid to acid may be accomplished without the use of an inverted funnel, which serves as a percolator, if very rapid boiling is maintained so that the two layers appear as one. The percolator permits milder conditions and less chance of loss of the acid layer in the condenser, since with its use less heating with little or no refluxing is sufficient. Also, some delay accompanies the return of a sharp interface when vigorous boiling substitutes for percolation.

The sensitivity and duplicability of turbidimetric measurements of quinine silicotungstate in  $0.03 \pm 0.002$  N sulfuric acid (Table II) compares favorably with the previous use of 0.03 N hydrochloric acid.<sup>2</sup> When both fluorometric and

turbidimetric determinations are desired on the 0.03 N sulfuric acid layer to which the alkaloid has been transferred, the latter should be run first since it is the less sensitive of the two and serves to indicate accurately the necessary factor of dilution for the application of the former. The lowest level of quinine which is measurable turbidimetrically requires at least a tenfold dilution before fluorometric measurement. Tenfold dilution with 0.1 N sulfuric acid reduces the acidity to 0.093 N. Quinine can be read accurately at this acidity against fluorometric standards which are customarily prepared in 0.1 N sulfuric acid. According to the study by Desha and associates<sup>18</sup> on the effect of hydrogen ion concentration on fluorescence, much greater differences in the concentration of sulfuric acid are permissible between standards and unknowns. Such permissible differences have been confirmed more recently by Valk.<sup>19</sup> Determination of the extracted quinine is not limited to the methods which have been selected in this study, but colorimetric or spectrophotometric methods should be applicable to the extracts.

#### SUMMARY

A rapid procedure is presented for the quantitative separation of quinine from biologic fluid or tissue by continuous extraction of a digested sample with chloroform. Specially designed apparatus which is simple in construction is described in various dimensions. The extractor represents an interchangeable adaptation of one proposed previously, so that the complete unit permits extraction with solvents of either greater or lesser density than water. The procedure enables the versatile selection of two or more established methods of analysis for simultaneous application to individual samples and stresses the value of this practice in metabolic studies since no specific method is available for quinine. The procedure has proved efficient and convenient during extensive use in animal work.

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#### REFERENCES

1. Kaiser, F. J.: *Methode zur Bestimmung des Chiningerhaltes in Blut*. *Rec. trav. chim.* 57: 117, 1938.
2. Prudhomme, R. O.: A New Colorimetric Method for the Determination of Quinine in Biological Fluids and Organs, *J. pharm. chim.* (9) 1: 8, 1940.
3. Kyker, G. C., Webb, B. D., and Andrews, J. C.: The Estimation of Small Amounts of Quinine in Blood and Other Biological Materials, *J. Biol. Chem.* 139: 551, 1941.
4. Kelsey, F. E., and Geiling, E. M. K.: The Micro Determination of Quinine in Blood and Tissues, *J. Pharmacol.* 75: 183, 1942.
5. Brodie, B. B., and Udenfriend, S.: The Estimation of Quinine in Human Plasma With a Note on the Estimation of Quinidine, *J. Pharmacol.* 78: 154, 1943.
6. Carol, J.: Quantitative Determination of Quinine by Absorption Spectrophotometry, *J. Assoc. Official Agr. Chem.* 26: 238, 1943.
7. Brodie, B. B., Udenfriend, S., Baer, J. E., Chenkin, T., and Dill, W.: The Estimation of Basic Organic Compounds and a Technique for the Appraisal of Specificity; Application to Cinchona Alkaloids, *J. Biol. Chem.* 158: 705, 1945.
8. Kyker, G. C., and Lewis, D. P.: The Micro Determination of Certain Alkaloids and Other Bases by Photometric Turbidimetry, *J. Biol. Chem.* 157: 707, 1945.
9. Marshall, P. B., and Rogers, E. W.: A Colorimetric Method for the Determination of Cinchona Alkaloids, *Biochem. J.* 39: 258, 1945.

10. Stimson, W. M., and Reuter, M. A.: The Spectrophotometric Estimation of Methoxy-Cinchona Alkaloids, *J. Am. Chem. Soc.* 68: 1192, 1946.
11. Dearborn, E. H., and Marshall, E. K.: The Degradation of Quinine in the Duck, Chicken, and Dog, *J. Pharmacol.* 85: 202, 1945.
12. Marshall, P. B.: The Absorption of Cinchona Alkaloids in the Chick and Its Relationship to Antimalarial Activity, *J. Pharmacol.* 85: 299, 1945.
13. Wasitzky, A.: Ein einfacher Mikroextraktionsapparat nach dem Soxhlet-Prinzip, *Mikrochemie* 11: 1, 1932.
14. Briggs, L. H.: Apparatus for the Extraction of Liquids With Immiscible Solvents of Greater Density, *Indust. & Engin. Chem. (Anal. Ed.)* 9: 250, 1937.
15. Wayman, M., and Wright, G. F.: Small Continuous Liquid-Liquid Extractors, *Indust. & Engin. Chem. (Anal. Ed.)* 17: 55, 1945.
16. Cori, C. F.: The Fate of Sugar in the Animal Body. I. The Rate of Absorption of Hexoses and Pentoses From the Intestinal Tract, *J. Biol. Chem.* 66: 691, 1925.
17. Alogdelis, S.: The Relationship of Quinine Metabolism and Vitamin B-Complex Deficiency, Master's Thesis, University of North Carolina, 1946.
18. Desha, L. J., Sherrill, R. E., and Harrison, L. M.: Fluorometry. II. The Relation Between fluorescence and Hydrogen-Ion Concentration, *J. Am. Chem. Soc.* 48: 1493, 1926.
19. Valk, A.: Studies in the Metabolism of Quinine, Doctoral Dissertation, University of North Carolina, p. 58, 1944.



## THE COUNTING OF BLOOD CELLS BY DARK-FIELD ILLUMINATION

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**M**ICROSCOPIC preparations are usually examined by bright-field illumination. The entire field of view is lighted, and preparations to be examined are shown against a light background. Objects are differentiated optically from their surrounding medium by their difference in refractive index, by natural coloration, or by the application of dyes.

In contrast to this is dark-field illumination in which objects appear as self-luminous structures on a dark background. This effect is obtained with the Abbe condenser by placing an opaque stop between the condenser and the source of light so as to exclude the central part of the light rays from the mirror, allowing only an external ring of light to pass to the condenser. The hollow cone of light thus formed illuminates brilliantly any object at its focus, and the object itself reflecting the light into the microscope appears self-luminous on a dark field.

In comparison to light-field, dark-field illumination has had few applications. Perhaps its most important use in medical science is for the detection of motile organisms such as the spirochetes of syphilis and trypanosomes in body fluids. It also should be of value for the enumeration of microscopic objects in counting chambers which, because of their small size, lack of color, or refractive index, are difficult to see with light-field illumination. A few possible applications are the counting of red blood cells, white blood cells, spinal fluid cells, blood platelets, bacteria, yeast, mold, plankton, and dust particles. Although the study of fresh blood cells on thin object slides by dark-field illumination is mentioned in Gage<sup>1</sup> and Gradwohl,<sup>2</sup> to the best of our knowledge, there is no reference in the literature concerning the counting of blood cells in counting chambers by this method of illumination. This is, no doubt, due to the required object slide thickness necessary for dark field. The object slide with the Abbe condenser and dark-field stop must be approximately 1.3 to 1.4 mm. in thickness, so that the apex of the hollow cone of light can be brought into coincidence with the object. It can be seen readily from the foregoing statement that if relatively thick chambers are used, this hollow cone of light would come to a focal point below the blood cells within the glass counting chamber, and the amount of light thus produced would be inadequate for illumination of the object. The solution to this problem is to increase the focal length of the Abbe condenser by removing its top lens, the numerical aperture of the condenser thus being reduced from 1.25 to 0.30. If the 43X, 0.65 N.A. objective is used, it is necessary also to decrease its numerical aperture by the use of a funnel stop. The method including preferable microscope accessories is described in detail.

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## EQUIPMENT

Although any biologic microscope with Abbe condenser, numerical aperture 1.25, can be used, a binocular medical microscope with 10X paired oculars is suggested for full appreciation of this dark-field method. If increased magnification is desired with the 10X, 0.25 N.A. objective, 15X oculars can be used. The source of illumination must be more intense than usually is required for light-field counting. A microscope lamp using a 115 volt, 100 watt Mazda bulb or a 6 volt, 108 watt ribbon filament bulb and equipment with a Corning Daylite filter and condensing lens furnishes ample illumination for use with either a monocular or binocular microscope. Two dark-field stops, 16 and 20 mm. in diameter, a funnel stop, a hemacytometer, and red and white pipettes complete the essential equipment.

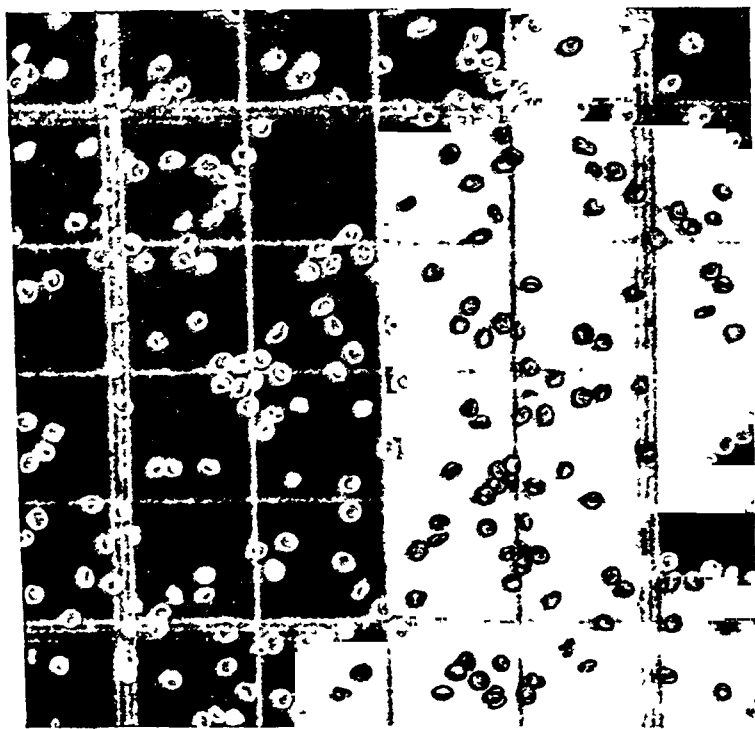


Fig. 1.—Red blood cells as seen with a 10X huygenian eyepiece and a 43X achromatic objective with funnel stop. Three-fourths actual size.

## METHOD

The usual dilution fluids, such as Hayem's solution and normal saline for erythrocytes and 1 to 3 per cent acetic acid (tinted with gentian violet) and 0.1 N hydrochloric acid for leucocytes, can be used. However, acetic acid is preferable to hydrochloric since it has a less destructive action on the cytoplasm of the white cells. This difference is more noticeable in dark-field than in light-field illumination. If, as previously recommended, a 115 volt, 100 watt bulb or a

6 volt, 108 watt bulb is used with a binocular microscope, the microscope lamp should be adjusted so as to produce a parallel beam of light. This can be done readily by directing the light beam at a wall at least six feet away and focusing the condensing lens until an image of the source is formed on the wall. The illuminator then can be oriented with reference to the microscope and the beam of light directed at the microscope mirror. A filter should be placed in the holder usually provided with most microscope lamps. This is necessary in order to eliminate all glare from the blood cells and counting chamber, as shown against the dark background. It was found that for this purpose a Corning Daylite filter was superior to the usual combination of a ground glass and blue filter. In order to bring the apex of the hollow cone of light on the blood cells in the counting chamber, the upper element of the Abbe condenser now is unscrewed and removed. A 16 mm. dark-field stop, usually supplied with most microscopes, is placed and accurately centered in the holder provided below the condenser. Using a combination of the low power objective (10X, 0.25 N.A.) and 10 or 15X oculars, the substage condenser now is racked up and down until the counting chamber ruling and blood cells appear white on a black background. It will be found that if the condenser is lowered slightly below this point, a very pleasing illusion of depth is obtained. This effect of depth is appreciated especially in the case of the white blood cells and is of value in distinguishing these cells from possible dust particles that may have been in the diluting fluid or on the counting chamber. If again the condenser is lowered below this point, blood cells appear dark against a light background. Since the counting chamber ruling also is light, this location of the substage condenser is not usually of value.

Dark-field illumination can also be used with the 43X, 0.65 N.A. objective, but its numerical aperture of 0.65 must be reduced by inserting a funnel stop. This stop is standard equipment and, although designed for reducing the aperture of the 97X, 1.25 N.A. objective, it is interchangeable. The size of the dark-field central stop at this magnification should be approximately 20 millimeters. As with the 10X objective, the condenser should be racked up and down until the counting chamber ruling and blood cells appear white on a dark background.

A modified form of dark-field illumination can be obtained by the substitution of Rheinberg color discs for the opaque stops. These discs can be prepared in the laboratory from colorless and red and green-colored gelatin or cellulose acetate, or fixed photographic film can be dyed to the desired shade. The central green disc must be 16 mm. for use with the 10X objective and 20 mm. with the 43X objective. It should be centered and cemented to either an outer colorless or red disc which must be approximately  $1\frac{1}{16}$  inches in diameter. Blood cells thus are shown as white objects on a green background when the central green disc is used in combination with an outer colorless disc, and red on a green background when a red disc is substituted for the outer colorless disc. If the latter combination is used, a ground glass filter should be substituted for the Corning Daylite filter. The results obtained are very spectacular but probably have no advantage over the usual dark-field method.

The location of the stops or Rheinberg discs above the lower element of the Abbe condenser will increase the brightness of objects and lines and will add to the ease with which optimum dark-field illumination can be obtained. Since there is no slotted ring provided at this location, it is necessary to construct a holder so designed that the stop or disc is at a location approximately 5 mm. above the condenser. If one expects to do a considerable amount of counting by dark-field illumination, it is believed that the superior results obtained will justify the special construction of the stop holder. The size of the stops or Rheinberg discs in this location should be approximately 13 mm. for the 10X, 0.25 N.A. objective and 16 mm. for the 43X, 0.65 N.A. objective.

#### SUMMARY AND DISCUSSION

A method has been described for the counting of blood cells in an all glass counting chamber (hemacytometer). This technique, modified as may be necessary, can be used for counting other objects previously mentioned. In order to increase the focal length of the Abbe condenser, 1.25 N.A., the upper lens of the condenser must be removed; if a 43X, 0.65 N.A. objective is used, a funnel stop is necessary to reduce its numerical aperture. Dependent on the location chosen, above or below the condenser, a 13 or 16 mm. stop or disc should be used with the 10X objective and a 16 or 20 mm. stop has proved satisfactory with the 43X objective. The size of the stop for either the 10X or 43X objective is not critical. It is possible that with counting chambers other than those used in this study better results may be obtained with stops slightly smaller or larger than those suggested. The brightness of the counting chamber ruling is dependent on the depth and width of the lines and also on the thickness of the counting chamber. For best results, the over-all thickness of the chamber should be 5 mm. or less and the ruling should be relatively deep and heavy. In place of opaque stops Rheinberg color discs can be used. Blood cells thus are shown as white or red objects on a green background.

#### REFERENCES

1. Gage, S. H.: *The Microscope*, ed. 17, Ithaca, N. Y., 1943, Comstock Publishing Co., Inc., p. 159.
2. Gradwohl, R. B. H.: *Clinical Laboratory Methods and Diagnosis*, ed. 2, St. Louis, 1938, C. V. Mosby Company, p. 309.

# CLOROX AND TERGITOL-JAVELLE WATER MIXTURE FOR ACID-FAST BACILLI CONCENTRATION

## A COMPARATIVE STUDY

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THE concentrated sputum examination for tubercle bacilli has largely superseded that of the plain smear. Because of the increased numbers of such examinations, a shorter more rapid technique was required. In recent years Clorox<sup>1</sup> has been used as the concentrating substance. Cameron and Castles<sup>2</sup> found this method superior to that of NaOH-alum and autoclaving methods, and we have similarly been impressed by its value in the past eighteen months. Certain difficulties were encountered, particularly regarding altered staining properties of the tubercle bacilli, rendering the slides difficult to read and interpret. We had previously used a tergitol-javelle water mixture, after its introduction by Petroff and Schain,<sup>3</sup> without such difficulties in staining properties. It was decided to evaluate again these two digestants in a parallel series to determine their comparative efficacy.

### METHOD OF STUDY

All sputa used in this study were collected routinely in bottles for a twenty-four to forty-eight hour period. After stirring thoroughly with wooden applicators each sputum was divided into two portions. One portion was treated with an equal volume of clorox, stirred with an applicator stick, and centrifuged in 15 c.c. tubes\* for ten minutes at 2,200 r.p.m. The clorox sputum mixture was not allowed to stand longer than ten minutes prior to centrifugation, as it was found that prolonged exposure (twenty minutes or more) rendered some organisms nonacid-fast or markedly impaired their staining properties. After decanting the supernatant fluid the centrifuge tubes were inverted for fifteen to twenty minutes over absorbent paper to allow for maximum drainage. The sediment was smeared and then stained by the Ziehl-Neelsen method.

The other portion of each sputum was treated with tergitol-javelle water mixture. This digestant was prepared in the manner recommended by Petroff and Schain.<sup>3</sup> Equal volumes of this digestant and sputum were mixed and agitated periodically with applicator sticks until digestion was completed. The incubation period employed by Petroff and Schain was omitted in this series. Digestion usually was completed within a few minutes. Some specimens, however, were very resistant and required as long as thirty minutes for homogenization to occur. Centrifugation was done in the same manner as employed in the clorox series. The decanted tubes were inverted over absorbent paper for fifteen to thirty minutes, a longer time being required in some cases to obtain maximum drainage. The smeared slides were stained in the previously mentioned manner.

No slide was considered negative until well over 100 fields had been examined. The method of reporting positive slides was based on the recommendation of the American Trudeau Society,<sup>4</sup> which is as follows:

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\*Sixteen-place, type 2, International Centrifuge.

Numerous (+++): Ten or more acid-fast bacilli in most oil immersion fields

Few (++) : Single acid-fast bacillus found in most oil immersion fields

Rare (+): Single acid-fast bacillus found in occasional oil immersion fields

In the case of 1 plus slides the number of bacilli found in the field examined were recorded.

### RESULTS OF THE STUDY

In the course of this study 171 sputum specimens were examined. One hundred specimens were found to be negative by both methods; seventy-one were found to be positive by one or both methods. The method employing tergitol-javelle water mixture gave eleven more positive slides than did the clorox method (Table I). With the method employing tergitol-javelle water mixture, 42 per cent of the slides were positive for tubercle bacilli. By the clorox method only 35 per cent were found positive. Thus the method using tergitol-javelle water mixture was superior by 7 per cent to the clorox method.

TABLE I. TOTAL NUMBER OF POSITIVE AND NEGATIVE SLIDES

NUMBER OF SPUTA EXAMINED	TERGITOL-JAVELLE WATER MIXTURE		CLOROX	
	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE
171	100 (58%)	71 (42%)	111 (65%)	60 (35%)

TABLE II. COMPARISON OF DEGREE OF POSITIVITY WITH BOTH METHODS

NUMBER OF POSITIVE SLIDES	TERGITOL-JAVELLE WATER MIXTURE			CLOROX		
	+++	++	+	+++	++	+
71 (Total positive specimens)	19	23	29	10	21	29
60 (Positive by both methods)	19	23	18	10	21	29

In Table II the breakdown of the positive slides into the degree of positivity is recorded. It is to be noted that there is a decided shift to lesser degrees of positivity with the clorox method.

In those specimens found to be 1 plus by both methods (nineteen cases), slightly over twice as many organisms were found per 100 fields by the method using tergitol-javelle water mixture as with the clorox method (Table III).

TABLE III. COMPARISON OF NUMBERS OF BACILLI IN 1 PLUS SLIDES (NINETEEN CASES)

METHOD	TOTAL FIELDS EXAMINED	NUMBER OF BACILLI FOUND IN FIELDS EXAMINED	AVERAGE NUMBER OF BACILLI PER 100 FIELDS
Tergitol-javelle	745	92	12.4
Clorox	1,108	57	5.1

### CONCLUSION AND SUMMARY

One hundred seventy-one sputum specimens were examined by both the clorox and the tergitol-javelle water mixture methods. Out of a total of seventy-one positive specimens the method using tergitol-javelle water mixture gave more positive results and exhibited better ability to concentrate the bacilli than did the clorox method. The treatment of the acid-fast bacilli with the mixture of

tergitol-javelle water does not show the impairment in staining that occasionally occurs when treated with clorox. For these reasons it was decided to abandon the use of clorox in favor of tergitol-javelle water for concentration purposes.

The writer wishes to express his appreciation to Dr. Allan Hurst for suggesting this study and for the aid received in the preparation of this paper.

#### REFERENCES

1. Oliver, J., and Reusser, T. R.: Rapid Method for the Concentration of Tubercle Bacilli, *Am. Rev. Tuberc.* 45: 450, 1942.
2. Cameron, G. M., and Castles, R.: Detection of Tubercle Bacilli in Sputum, Application of the NaOH-Alum Method, the Clorax Method, and the Direct Smear, *J. LAB. & CLIN. MED.* 31: 361, 1946.
3. Petroff, S. A., and Schain, P.: Sputum Examination; Additional Data Pertaining to Digestion and Staining, *Quart. Bull., Sea View Hosp.* 5: 183, 1940.
4. American Trudeau Society: Report of the Committee on Standard Laboratory Procedure, *Am. Rev. Tuberc.* 50: 465, 1944.

# MICROPRECIPITATING UNIT FOR BLOOD AND TISSUE PROTEINS

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IN THE course of microanalysis of blood and tissues it is often necessary to precipitate proteins. This is usually done by mixing the blood and the protein-precipitating fluid, placing the mixture in a capillary tube, sealing, and centrifuging. The supernatant fluid is finally separated from the precipitate by cutting the tube just above the level of the precipitate. The method described in Methods enables one to carry out all operations within the same pipette. The drawing of blood and the precipitation of proteins can easily be done in a patient's room, and the tube can be set aside for centrifuging upon return to the laboratory.

## METHODS

The pipette\* illustrated in Fig. 1 consists of a capillary tube used for measuring the blood and a diluting bulb whose volume is equal to five or ten times that of the capillary tube. The bulb end is sealed to a standard taper male joint and is fitted with a standard taper cap. Rubber bands placed on the hooks serve to hold the cap in place during centrifugation. Blood is drawn into the

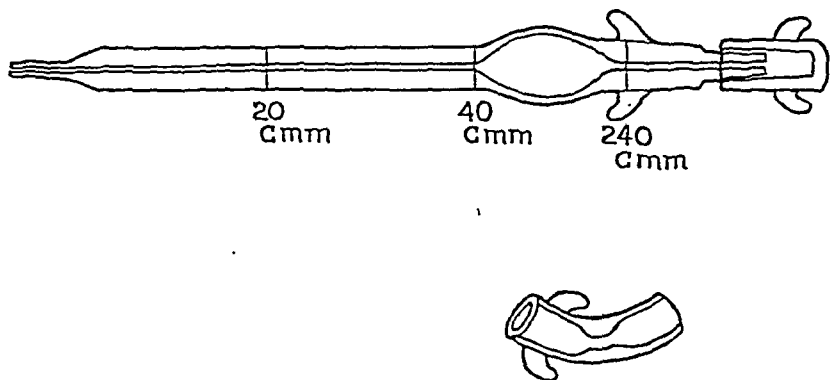


Fig. 1.—Top, a blood-precipitating pipette fitted with standard taper joint and cap. Bottom, a standard taper female-female adapter.

pipette to the 20 c.mm. mark. The protein-precipitating fluid is then drawn into the pipette to the 240 c.mm. mark. After mixing, a drop is blown out of the pipette to remove the unmixed protein precipitating fluid which is present within the capillary portion of the pipette. The cap is put on and fixed in position with a rubber band; the pipette may be put aside for a time at this stage. After centrifugation at appropriate speed, the cap is removed.

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\*The pipette was made by James D. Graham, University of Pennsylvania School of Medicine, Philadelphia, Pa.



The volume of the cap is arranged so that all the precipitate can be contained within the cap, the pipette proper containing only blood filtrate. If the volume of the cap is 2.25 times that of the blood used, the cap will contain all the blood precipitate when tungstic acid is used as the precipitating agent. If a 5 per cent solution of trichloroacetic acid is used, the volume of the cap should equal the volume of blood used. The female-female adapter shown in Fig. 1 is used in place of the cap to transfer the blood filtrate to the microcolorimetric reaction-vessel described in another paper (page 215).

If the precipitate is desired, the remaining supernatant fluid in the cap can be drawn off with a capillary tube and the precipitate can then be removed from the cap.

# A SIMPLE APPARATUS FOR QUANTITATIVE MICROCOLORIMETRIC ANALYSIS IN FINAL VOLUMES OF 0.15 c.c.

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LOWRY and Bessey<sup>1</sup> have reported that accurate measurements may be made in the Beckman spectrophotometer, on volumes of 0.1 c.c., by using a special colorimeter cell. I have found also that the Lumetron photoelectric colorimeter could be used for this purpose if it were equipped with a galvanometer of the multiple reflection type. Special 10 by 3 mm. cells were used. White blood cell diluting pipettes were used for carrying out the colorimetric reactions.

The technique described herein simplifies the microcolorimetric procedures. All volume measurements, heating, and color development can be carried out within one tube.

## APPARATUS

A tuberculin syringe (1 c.c. with graduations of 0.01 c.c.) is used to control the volume within the reaction pipette. To facilitate smooth operation the plunger is fitted with a spring clip. A Shick-Vim syringe can be used in place of the tuberculin syringe. A convenient reaction-vessel\* for microcolorimetry (Fig. 1) consists of a capillary tube with a capacity of 30 c.mm. and a dilution bulb whose volume is 150 c.mm. The capillary tube, which must be of constant internal diameter, is graduated into 50 or 100 divisions, so that fractional volumes may be measured. The bulb end of the pipette is fitted with a standard taper female joint,† whereas, the pipette end is fitted with a standard taper male joint. A standard taper cap fits over this pipette end for sealing. A glass bead sealed in the mixing bulb facilitates mixing.

The pipette and syringe are mounted by clips at about a 30 to 45 degree angle (Fig. 2). A three-way spinal puncture stopcock (Yale B-D L/S 2) is placed between the syringe and the reaction pipette. The purpose of this stopcock is to facilitate removal and replacement of a partially filled reaction pipette from the syringe. When reinserting a partially filled reaction pipette into the stopcock, the latter should be open to permit flow of air from the side tube; otherwise fluid may be forced out of the tip of the pipette as it is inserted into the stopcock. With the pipette in place, however, the stopcock should be turned so that the pipette communicates only with the syringe.

The joints should be greased and the syringe oiled to prevent leakage of air into the system.

From the Department of Anatomy, Western Reserve University School of Medicine, Cleveland, Ohio, and the Marine Biological Laboratory, Woods Hole, Mass.

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\*The reaction-vessel was made by James D. Graham, University of Pennsylvania School of Medicine, Philadelphia, Pa.

†Because of greater simplicity of manufacture, the pipette now supplied by J. D. Graham is fitted with a male joint at the bulb end and a male-female adapter inserted on the three-way stopcock.

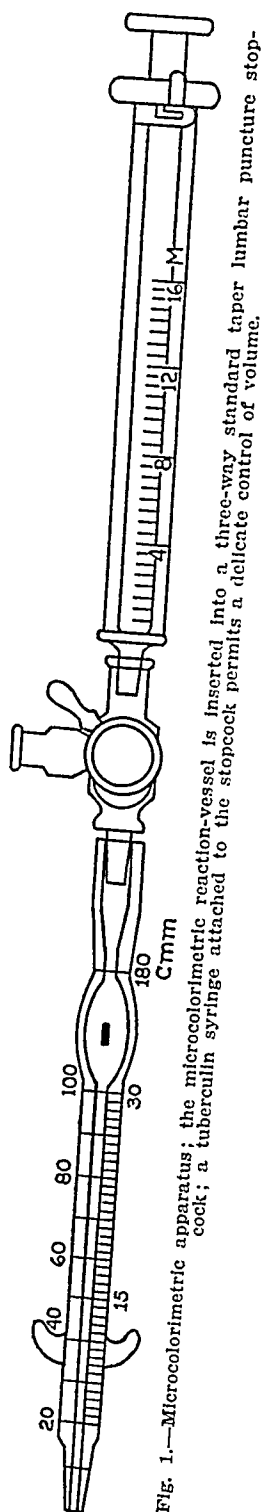


Fig. 1.—Microcolorimetric apparatus; the microcolorimetric reaction-vessel is inserted into a three-way standard taper lumbar puncture stop-cock; a tuberculin syringe attached to the stopcock permits a delicate control of volume.

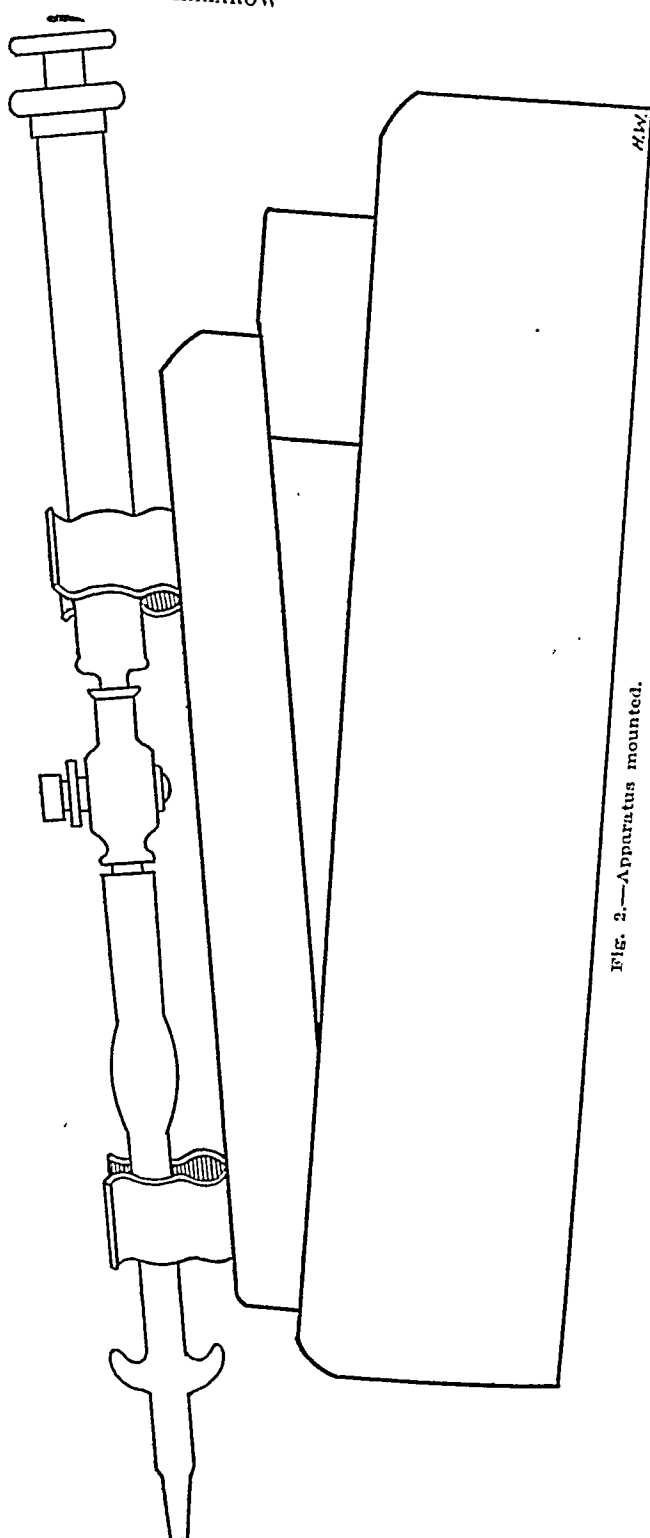


Fig. 2.—Apparatus mounted.

A vessel for the addition of reagents is shown in Fig. 3. It has a bulb containing about 5 c.c. and is fitted with a standard taper female joint and a stopcock to control the fluid in the bulb.

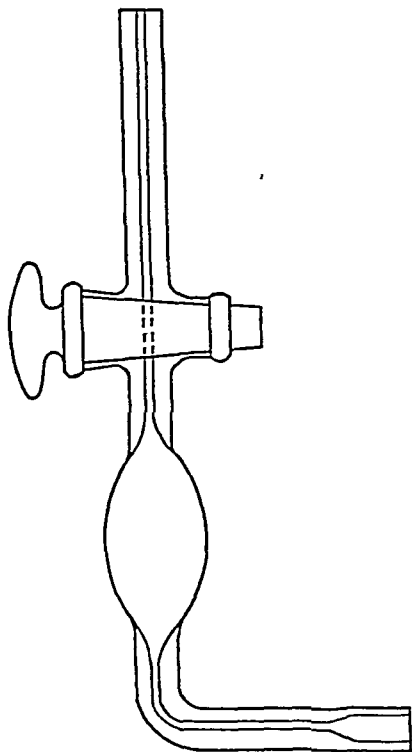


Fig. 3.—Reagent-addition vessel.

#### METHODS

In carrying out the colorimetric procedure blood filtrate or tissue extract is placed in the reaction-vessel. For this purpose the female-female adapter is placed on the male joint of the blood-precipitating pipette described in another paper<sup>2</sup> (page 213), and this in turn is connected with the reaction-vessel. The blood filtrate is drawn into the tube a little past the 15 c.mm. mark, because removal of the blood precipitating pipette causes the level of liquid in the colorimeter reaction tube to change slightly. By means of the syringe, the volume is adjusted to exactly the 15 c.mm. mark. A small bubble of air is then drawn into the capillary in preparation for adding the next reagent. If blood sugar were being determined by the Folin-Malmros<sup>3</sup> method, one volume of alkaline ferrieyanide solution would be added next. For this purpose the reagent-addition vessel (Fig. 3) is connected to the male joint of the reaction-vessel. The stopcock is opened and by means of the syringe the liquid is drawn into the pipette slightly above the 15 c.mm. mark. The stopcock connecting the syringe and the reaction-vessel is closed, the stopcock of the reagent-addition vessel is closed, and then the latter vessel is removed. The three-way stopcock

that connects the syringe and the pipette then is opened, the volume is adjusted to exactly 15 c.mm., and the alkaline ferricyanide solution is drawn into the bulb. Mixing with the blood filtrate can be accomplished by alternately forcing the liquid into the pipette and drawing it back into the bulb. A cap is placed on the male joint of the reaction-vessel, and this is secured in place with a rubber band; the vessel then is removed from the stopcock. Further mixing can be accomplished, if desired, by rotating the pipette while in a vertical position. (The pipette must not be inverted, for if the fluid reaches the upper capillary, later dilutions cannot be made with great accuracy.) The tubes are placed, at an angle of 30 to 45 degrees, in a water bath designed to heat only the bulbs of the pipette. If the cap and capillary portion of the pipette are also heated, the air within them expands and forces its way out past the liquid in the bulb and then out the bulb end. On cooling, however, the air contracts and the liquid is sucked from the bulb into the capillary and cap.

After cooling, the pipette is replaced upon the stopcock, the cap is removed, and two volumes of a solution of ferric gum ghatti are measured and then sucked into the bulb. The cap is reinserted, the pipette is removed from the rack, and the contents are mixed by rotation in the vertical position. After standing twenty minutes, the reaction-vessel is replaced, distilled water is added to the 180 c.mm. mark, and the contents of the bulb are mixed by shaking. The drop of water contained in the capillary tube is blown out. Then an adapter is inserted on the male joint, the contents are transferred to a microabsorption cell, and the extinction is measured in a photoelectric colorimeter.

#### DISCUSSION

By using standard methods and a volume of 150 c.mm., a large number of colorimetric procedures can be carried out on samples of tissue weighing only 1 mg. or on a few cubic millimeters of blood. The procedure is simple and could be employed routinely in the determinations customarily carried out in the clinical laboratory. It should prove especially useful in pediatric practice, since a few cubic millimeters of finger blood will prove sufficient for most chemical procedures.

If 50 c.mm. (about 1 drop) of blood are available for chemical analysis, the final volume for colorimetry need not be limited to 0.15 c.c. but can be carried out on final volumes of 1.0 cubic centimeter. Sufficient blood filtrate is obtained from one drop of blood to carry out several chemical determinations. By applying the technique used by Shock and Hastings,<sup>4</sup> it should be possible to substitute a sector of a regular cylinder for part of the bulb, and in this case the pipette itself could be used for the colorimeter tube. The volumes of the colorimetric reaction—vessel may be reduced if desired for only 50 c.mm. are required for use with the Beckman microcells.

The accuracy of this procedure has been found to approach that of ordinary macrocolorimetric procedures. Inasmuch as all volumes are measured within the same pipette, it is necessary only to determine the dilution-factor of the bulb for each pipette. This can be done simply by measuring 15 c.mm. of a known colored solution in the pipette, diluting it to 180 c.mm. and mixing. The

Distilled water contained in the capillary is blown out and the contents of the bulb are then placed in the microabsorption cell. The transmission of light is measured and the precise dilution of the 15 c.mm. is thus determined in the pipette under the conditions of use. The absolute volume of a given pipette makes relatively little difference in the determination.

The microcolorimetric procedure can be carried out as rapidly as an ordinary macroprocedure. The small amount of blood required (finger puncture), the simplicity of the method, and the economy of table space and dish washing—all serve to make this procedure highly desirable in ordinary clinical chemistry. This technique should be most useful in special research projects where only small amounts of material are available.

#### SUMMARY

A simple apparatus is described which is useful in colorimetric analysis employing small final volumes (0.15 c.c. or less). Samples of blood or other tissues of 0.001 c.c. or 1 mg. are adequate for most colorimetric procedures using this technique.

#### REFERENCES

1. Lowry, O. H., and Bessey, O. M.: The Adaptation of the Beckman Spectrophotometer to Measurements on Minute Quantities of Biological Materials, *J. Biol. Chem.* 163: 633, 1946.
2. Lazarow, A.: Microprecipitating Unit for Blood and Tissue Proteins, *J. LAB. & CLIN. MED.* 32: 213, 1947.
3. Folin, O., and Malmros, H.: An Improved Form of Folin's Micro Method for Blood Sugar Determinations, *J. Biol. Chem.* 83: 115, 1929.
4. Shock, N. W., and Hastings, A. B.: A Micro Technique for the Determination of the Acid Base Balance of the Blood, *Proc. Soc. Exper. Biol. & Med.* 26: 780, 1929.

## BOOK REVIEWS

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**Penicillin—Its Practical Application.** Edited by *Sir Alexander Fleming*, M.B., B.S., F.R.C.P., F.R.C.S., F.R.S., Professor of Bacteriology, the University of London, St. Mary's Hospital, London. The Blakiston Company, Philadelphia, 1946. Price \$7.00. Cloth with 380 pages, 59 illustrations.

Since all of the twenty-eight contributors to this book, prepared under the editorship of Sir Alexander Fleming, are British, it constitutes a useful compendium of the investigative work and clinical applications of penicillin by British workers, much of which may not be familiar to American physicians.

The first section, totaling 104 pages, is devoted to the general aspects of the subject and consists of six chapters on the history and development, chemistry and manufacture, pharmacy, pharmacology, bacteriological control, and methods of administration of penicillin. It is needless to state that the chapter on the history and development of the compound, written by Fleming himself, is outstanding. The chapter on the chemistry and manufacture of penicillin is likewise comprehensive, although the different penicillins (F, G, X, and K) are but barely mentioned and much of the space, with numerous illustrations, devoted to methods of manufacture could have been better expended on more comprehensive discussions of the pharmacology and bacteriological aspects of the subject in view of their greater clinical importance. The pharmacy of penicillin is devoted exclusively to methods employed by the British Pharmacopeia, and while Fleming adequately describes his own methods for the detection and assaying of penicillin in the blood, urine and other body fluids, the Oxford cup and various serial dilutions methods are so briefly and inadequately described as to be of but little or no value to laboratory workers. One may also regret that so little is given on the antimicrobial activity of penicillin *in vitro* and *in vivo* or likewise, on the subjects of natural and so-called acquired resistance of bacteria to penicillin, with methods for determining the susceptibility of organisms to the compound *in vitro* which are so important in relation to penicillin therapy.

The second section, 21 chapters totaling 257 pages, is devoted to the clinical aspects of penicillin in the prophylaxis and treatment of disease. Some of these chapters, especially those devoted to the prophylactic use of penicillin, the treatment of wounds and gangrene and the treatment of dental and oral infections, are satisfactory. All of the chapters summarize the clinical experience of British physicians and surgeons, and some diseases are discussed in a very superficial fashion due to a lack of experience; this is especially true of the venereal diseases and particularly of syphilis. Since the book was written by a large group of contributors, there is much useless duplication of material with special reference to methods of administration. For example, there is a chapter on chest infections and another on chest surgery which could have been combined; likewise, there is a chapter on orthopaedic surgery and fractures and another on osteomyelitis which could also have been advantageously combined. Why a chapter on penicillin and the general practitioner was included is difficult to understand, particularly because of its sketchy character and the fact that practically all of the material is covered in other chapters.

While references to the literature of American contributions to penicillin are no slighted, the value of the book from the reference standpoint is sharply limited. Thus the chapters on penicillin in the treatment of chest infections and on the treatment of brain and meningeal infections have no references at all, those on methods of administration and on orthopaedic surgery and fractures have but one each, that on the chemistry and manufacture of penicillin only two, and that on hand infections only three.

**Modern Development of Chemotherapy.** By *E. Havinga, H. W. Julius, H. Veldstra, and K. C. Winkler.* Elsevier Pub. Co., Amsterdam-New York, 1946. Price \$3.50. Paper with 175 pages.

In spite of the ever-growing burden of oppression and starvation in Holland due to the recent war, research was continued intensively in all directions. Some of the results were published in Dutch periodicals but most of them, for obvious reasons, were kept a secret during the war. The present monograph is one of a series summarizing this research work in different fields now being published, with the hope that they will further intensify the interest shown by the allied nations in the Netherlands and the whole-hearted preparedness of this courageous nation to contribute to the progress of mankind.

The present monograph clearly and concisely summarizes the investigations of Havinga and Veldstra in Amsterdam and of Julius and Winkler in Utrecht on the following: The mechanism of action of the sulfonamide compounds and p-aminobenzoic acid; chemical investigations on the synthesis and activity of sulfonamide derivatives and of related compounds; pharmacological, immunological, and clinical investigations on sulfonamide compounds; as well as a brief chapter bearing upon investigations on antibiotic agents in mycotherapy, with special reference to expansine. Foreign literature is referred to only in so far as it has a direct bearing on the Dutch research work.

JOHN A. KOLMER, M.D.

**Renal Hypertension.** By *Eduardo Braun-Menendez, Juan Carlos Fasciolo, Luis F. Leloir, Juan M. Munoz, and Alberto C. Taquini,* Institute of Physiology, Faculty of Medical Sciences, and Institute of Cardiology, V. F. Greg Foundation, Buenos Aires, Argentina. Translated by *Lewis Dexter, M.D.,* Harvard Medical School and Peter Bent Brigham Hospital, Boston, Mass. Charles C Thomas, Publisher, Springfield, Ill., 1946. Price \$6.75. Cloth with 451 pages.

The authors' purpose is to describe and evaluate studies in experimental hypertension made during the past fifteen years. Topics such as the production of hypertension in animals, the renal vasopressor system, the roles endocrine and nervous influences play, and the possible correlations with hypertension in human beings are considered at length. An excellent bibliography follows.

The reviewer does not believe that present evidence justifies the strong views advanced by the authors on many points.

Thus, renal ischemia is apparently accepted as the initiating and common factor in experimental, renal, and clinical hypertension, although a close reading of the text shows no thoroughgoing proof of the assumption but a good deal of evidence to the contrary. The morphologic evidence that early in the disease hypertensive renal arteriolar sclerosis is often absent is disregarded, so the facts are whipped into a sort of ischemic party line. The authors subscribe to the notion that angiotonin (hypertensin) is the cause of the rise of arterial pressure in experimental and clinical hypertension. In so doing they necessarily underemphasize the failure to demonstrate such a substance in the blood of hypertensive dogs or human beings.

Examples can be multiplied and only serve to show that this field of investigation is far from static. Since the authors' assurance is not shared by most investigators, their statements might well mislead those introduced to the subject by a reading of this book. Expression of emotional preferences perhaps too often occurs for writing of this sort.

The book is well made and the translation good. It is recommended as a reference text for those engaged in the study of experimental renal hypertension.

ROBERT BIRCHALL.



**Peripheral Vascular Diseases.** By *Edgar F. Allen*, B.S., M.A., M.D., M.S. in Medicine, F.A.C.P., Division of Medicine, Mayo Clinic, Associate Professor of Medicine, Mayo Foundation, Graduate School, University of Minnesota, Diplomate of the American Board of Internal Medicine; *Nelson W. Barker*, B.A., M.D., M.S. in Medicine, F.A.C.P., Division of Medicine, Mayo Clinic, Associate Professor of Medicine, Mayo Foundation, Graduate School, University of Minnesota, Diplomate of the American Board of Internal Medicine; and *Edgar A. Hines, Jr.*, M.D., B.S., M.A., M.S. in Medicine, F.A.C.P., Division of Medicine, Mayo Clinic, Associate Professor of Medicine, Mayo Foundation, Graduate School, University of Minnesota; with Associates in the Mayo Clinic and Mayo Foundation. W. B. Saunders Company, Philadelphia, 1946. Price \$10.00. Cloth with 871 pages and 386 illustrations.

This book is by far the most extensive on peripheral vascular disease which has been published. There is a thorough review of what is known of disease affecting peripheral arteries, veins, and lymphatics. However, hypertension and vascular diseases of the central nervous system have not been treated because they are considered as special subjects and are beyond the scope of the present work.

Well-documented pathologic and clinical descriptions, supplemented by discussions of pathologic physiology, are offered for each vascular disease. Chapters on nailfold capillaries, the physiologic significance of sweating, the scalenus anticus syndrome, glomus tumors, and others were contributed by other authors. These chapters broaden the entire work. The surgical treatment of certain peripheral vascular diseases is considered in the final chapter. The medical treatment of such disorders is extensively reviewed. In the preface the authors stated that they would not hesitate to express their own opinions, particularly regarding controversial points of therapy. In what is largely an impartial review of this subject, it seems that their reasons for rejecting certain forms of treatment are usually made clear.

The book will largely fulfill its two goals, to aid the physician and student in the study and care of patients with peripheral vascular disease and to emphasize that "there remains a vast and fruitful field for research in peripheral vascular diseases."

JOHN R. SMITH.

## BLOOD TRANSFUSION STUDIES

### III. THE RELATIONSHIP OF HEMOGLOBINEMIA AND OF THE PH OF THE URINE TO RENAL DAMAGE PRODUCED BY INJECTION OF HEMOGLOBIN SOLUTIONS INTO DOGS

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**H**EMOGLOBINURIA occurring after hemolytic transfusion reactions has been a major problem in the use of whole blood transfusions since their inception. In 1667 Jean Baptiste Denys<sup>13</sup> first described the sequelae of a transfusion of incompatible whole blood (sheep blood). It is well known that serious reactions still occur after transfusion in spite of tests for compatibility, etc. There have been a large number of reports of anuria and death from transfusions since 1900. Hesse<sup>50</sup> received answers to questionnaires sent to 1,700 clinics and hospitals all over the world. Two hundred seventeen cases of hemolytic shock following transfusions were reported. Out of 200 cases in which definite statements were made as to the outcome, 105 of the patients died, or a mortality rate of 52 per cent. Other authors<sup>13, 21, 45, 68</sup> have reported small series of cases with comparable mortality rates. Hesse<sup>49</sup> also summarized the information available on the use of universal donors blood in the transfusion of patients of other groups and found twenty deaths occurring in a series of forty-eight reactions. Other isoagglutinins such as the anti-Rh agglutinin were not excluded in this group of cases, so high titers of agglutinins in the donors plasma cannot be incriminated in all of these cases. Hardin<sup>47</sup> compared two series of transfusions in the Army Medical Service. There was no difference in the reaction rates between the 7,299 transfusions of group specific blood and the 9,392 transfusions of universal donor blood. Blackwater fever is the most important cause of death of all patients with hemoglobinuria, for there are a large number of cases each year in tropical areas, and the mortality rate is approximately 25 per cent.<sup>38, 65, 79</sup> Recovery from either condition may occur after virtual anuria or oliguria of as much as a week's duration.<sup>8, 15, 20, 31, 41, 45, 56, 66, 68, 82</sup>

*Pathology of the Renal Lesion in Clinical Hemolytic Conditions.*—The mechanism of renal damage has been the subject of many pathologic studies in the past. Werner<sup>88</sup> and Barratt and Yorke<sup>8</sup> demonstrated that the kidneys of patients dying from blackwater fever contained granular material in the lumina of the tubules and attributed the urinary suppression to mechanical blockage of the tubules. Ayer and Gauld,<sup>5</sup> Baker and Dodds,<sup>6, 7</sup> Bordley,<sup>13</sup> Daniels and associates,<sup>21</sup> DeGowin and co-workers,<sup>22-24</sup> Goldring and Graef,<sup>45</sup> and Lindau<sup>60</sup>

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have contributed much to the study of clinical cases of hemolytic transfusion reactions and the pathologic study of the renal damage produced. Very little attention has been given to hemoglobinemia. More surprising still, hemoglobinuria itself is not mentioned in some cases and is mentioned in a cursory manner in most reports.

The renal lesions after transfusion reactions have been compared with those in other hemoglobinuric states, namely, blackwater fever,<sup>5, 60</sup> acute hemolytic anemias from drug administration,<sup>37, 83</sup> burns,<sup>81</sup> and crush injuries.<sup>16</sup> March hemoglobinuria,<sup>44</sup> paroxysmal nocturnal hemoglobinuria,<sup>46, 52</sup> and paroxysmal cold hemoglobinuria<sup>64</sup> usually do not cause renal damage. Possible explanations for this will be discussed later. DeGowin and co-workers,<sup>22, 24</sup> Lindau,<sup>60</sup> Bordley,<sup>13</sup> and others<sup>18, 21, 45, 74, 83, 90, 92</sup> agree in all essential details about the pathologic findings. There are degenerative changes in the tubular epithelium, especially of the loops of Henle and distal convoluted tubules, interstitial edema in the corticomedullary portion, granules of hemosiderin in the cytoplasm of the cells of the proximal and distal convoluted tubules, and the appearance of a variable number of deeply pigmented casts in the lumina of the loops of Henle, distal convoluted tubules, and the collecting tubules. The degenerative tubular changes vary from mild vacuolization to necrosis and desquamation of cells. Calcium deposits in the degenerated tubules and definite signs of regeneration of epithelial cells have been found.<sup>45</sup>

The importance of the pigment casts has been a major point of dispute among students of the pathology of transfusion reactions and blackwater fever. Baker and Dodds<sup>6</sup> are of the opinion that the casts cause obstruction of the individual nephron and, hence, cause renal insufficiency. Many other investigators<sup>55, 74, 85, 91, 92</sup> agree with this concept, but there are some who believe that the cast formation is only secondary to the tubular epithelial degeneration and not the cause of renal insufficiency.<sup>5, 25, 45, 60</sup> In their study of nine cases DeGowin and co-workers<sup>24</sup> concluded that several patients had evidence of renal tubular obstruction sufficient to cause uremia but that most patients had evidence of tubular epithelial cell necrosis and interstitial edema as the predominant lesion. Thus, they thought that there are several factors which operate in cases of hemolytic transfusion reactions in human beings. Others<sup>13, 18, 21</sup> have also taken this intermediate position.

The liver is likewise involved in cases of hemolysis from any cause.<sup>45, 60</sup> There is a variable amount of central necrosis of the lobules of the liver. In some cases of blackwater fever and of transfusion reaction this is very severe, with the pathologic appearance of acute atrophy of the liver. The reticulo-endothelial cells of the spleen, liver, and lymph nodes contain a large number of hemosiderin granules.

*Experimental Production of Renal Damage by Injection of Hemoglobin Solutions.*—Many attempts to reproduce the renal damage in experimental animals have been partially or completely successful. Ponfick<sup>76</sup> in 1875 was the first one to attempt to reproduce the lesions occurring in blackwater fever. He found that the injection of heterologous blood into dogs, cats, and rabbits resulted in hemoglobinuria and the deposition of pigmented casts in the tubules of

the kidney. Levy<sup>59</sup> injected pure hemoglobin solution and both hemologous and heterologous blood cells into rabbits. He observed that the epithelium of the proximal convoluted tubules became swollen but retained its staining quality. Casts appeared in the loops of Henle and the collecting tubules. In some tubules the epithelium was entirely denuded.

Yorke and Nauss<sup>54</sup> injected homologous hemoglobin solution into rabbits and found that unless the rabbits had been taking a dry diet before the injection and unless the hemoglobin solution was concentrated anuria did not occur. The largest dose used by them was 13 Gm. at a single injection. The renal pathology was reported to be like that of blackwater fever resulting in urinary suppression. Barratt and Yorke<sup>8</sup> showed that if hemolyzed blood were centrifuged to remove the stroma of the red cells much larger amounts were then tolerated by rabbits. Sudden death resulted during or immediately after injection when the stroma was not removed. Other authors<sup>6, 12</sup> have had similar experiences. Levy,<sup>59</sup> however, was unable to produce any renal lesions by injecting erythrocyte stroma alone.

Baker and Dodds<sup>6</sup> reported results of three rabbit experiments and gave very incomplete protocols as to dose, pH of urine, and details of pathology. When the injected rabbits were getting a diet with an acid ash, they found that the urine was brown in color and contained much sediment; however, when the rabbits were getting an alkaline ash diet of green vegetables, the urine was red and clear. The acid urine contained methemoglobin and oxyhemoglobin, but the alkaline urine contained only oxyhemoglobin. They concluded that intravascular hemolysis resulting in suppression of urine caused blockage of the renal tubules only when the urine is acid.

DeGowin and co-workers<sup>23, 24</sup> injected hemolyzed dog erythrocytes into a number of dogs under various experimental conditions. Some of the dogs were fed an alkaline ash diet and the others a cooked meat diet with 8.0 Gm. of ammonium chloride daily. The doses of hemolyzed erythrocytes were measured in terms of the volume of packed red blood cells (unwashed) and ranged from 3.0 to 35.0 c.c. per kilogram of body weight with an average of 10 c.c. per kilogram. When the urine was alkaline, four dogs received thirty-two transfusions of more than 10 c.c. of hemolyzed red blood cells per kilogram but did not develop fatal renal insufficiency; however, when the urine was acid, seven out of twelve dogs given comparable doses of hemolyzed blood cells died of uremia, frequently with terminal rigidity and convulsions. Three died after a single transfusion, and four dogs died from uremia after an injection of 9.5 to 10 c.c. of hemolyzed packed red blood cells per kilogram. DeGowin and co-workers also gave small doses of hemoglobin (2 c.c. of packed cells per kilogram body weight) daily for thirteen days to a dog with acid urine and to one with alkaline urine; they found no evidence of renal damage in spite of continuous hemoglobinuria. The kidneys of the dogs dying from uremia had many large casts in the loops of Henle and distal convoluted tubules and degeneration or necrosis of the tubular epithelium in some areas. Several of the dogs with neutral or alkaline urine during injections were sacrificed when in good health. There was no evidence of cast formation or damage to the renal epithelium in these dogs.

Wakeman and associates<sup>55</sup> were able to produce anuria and death in dogs by intraperitoneal injections of large doses of hemoglobin solutions. The pathologic appearance of the kidney was similar to that noted by DeGowin and co-workers. Many others have failed to produce renal damage but have used much smaller doses and have paid little or no attention to this fact. Foy and associates<sup>40</sup> have criticized DeGowin's work stating that extrarenal factors of dehydration, hypochloremia, or acidosis probably accounted for the results obtained in acidified animals. However, DeGowin and co-workers specifically state that their animals were maintained in good condition on the acid diet for months without losing weight and that the carbon-dioxide combining power and plasma chloride levels were within normal limits before injecting hemoglobin solutions. The vomiting occurred only after animals had been injected. Some of these animals developed hypochloremia and alkalosis from vomiting after uremia had set in. In one of Bordley's cases<sup>13</sup> hypochloremia was not a feature in spite of the development of uremia. DeGowin and Baldrige<sup>22</sup> found normal carbon-dioxide combining power and chloride concentrations in their patients at the time of transfusion reactions. In the clinical history of patients with urinary suppression due either to transfusion reaction or to blackwater fever, the oliguria or anuria usually developed before any dehydrating factors such as vomiting could occur. A patient with blackwater fever reported by Wakeman and associates<sup>55</sup> had moderate depletion of chlorides on the day following development of hemoglobinuria. Nausea and vomiting in this patient were severe.

Bing<sup>11</sup> used solutions of oxyhemoglobin and methemoglobin made from crystals of the respective pigments instead of hemolyzed blood cells as used by most other investigators. However, the largest dose of hemoglobin used was 13.0 Gm., or a dose about one-third of the average amount used by DeGowin. Bing made a careful study of tubular and glomerular function. He was able to produce profound renal damage in dogs rendered acidotic by large doses of ammonium chloride following injection of 3.5 to 13.5 Gm. of methemoglobin solutions. Marked tubular damage and only rare pigment casts were observed microscopically in the kidneys of these dogs. Dogs with a normal alkali reserve did not develop any decrease in renal function when 5.5 to 8.1 Gm. of methemoglobin were injected. No decrease in function was observed in either normal or acidotic dogs when oxyhemoglobin solutions were injected. Bing produced an actual acidosis and not just an acid urine.

Aubertin and co-workers<sup>4, 58</sup> were able to produce varying degrees of renal insufficiency in dogs by the injection of varying doses of hemoglobin solutions after preliminary bleeding of the dogs. The largest doses uniformly caused severe renal injury or death from anuria. Those dogs with transient oliguria recovered completely, while those with longer-standing oliguria developed chronic renal insufficiency and death. In the pathologic material they found many pigment casts and were thus led to stress the obstruction of the tubules. Epithelial degeneration occurred also. No attention was given to the reaction of the urine.

*The Mode, Site, and Amount of Hemoglobin Excretion by the Kidney.*—Many investigators have studied the mode and site of excretion of hemoglobin by the kidney and the renal threshold for hemoglobin in man and experimental animals. Bieter<sup>10</sup> showed that hemoglobin was not excreted by the aglomerular toadfish but was excreted by the catfish and the eel in comparable doses. Doubling the dose of hemoglobin failed to produce hemoglobinuria in the toadfish. Albuminuria could not be produced in the toadfish either. Webster and associates<sup>56</sup> were unable to produce hemoglobinuria in frogs by perfusing the renal portal system with hemoglobin solution, but they were able to produce it by perfusing the aorta of the same frog preparations. Newman and Whipple<sup>70</sup> showed that repeated injections of hemoglobin in doses below the minimal renal threshold for excretion resulted in no deposit of iron pigment in the convoluted tubule cells. Lison<sup>61</sup> injected hemoglobin solution directly into the tubule lumen and found absorption of the hemoglobin granules into the tubule cells. He also ligated the renal artery of frogs and injected hemoglobin solution. No hemoglobin was excreted by the kidney and no granules of pigment appeared in the tubule cells. Yuile<sup>96</sup> agrees that hemoglobin escapes only by way of the glomerulus. The renal thresholds for hemoglobin can be explained on the same basis as thresholds for glucose and other substances, namely, that the tubule cells reabsorb all hemoglobin excreted up to a certain level.

Monke and Yuile<sup>69</sup> determined the simultaneous plasma clearance rates for creatinine and hemoglobin when plasma hemoglobin concentrations were about 250 mg. per cent. Hemoglobin clearance was found to be 3 per cent of that of creatinine. They found renal thresholds in a large series of dogs to vary from 80 to 150 mg. per cent. Yuile and Clark<sup>98</sup> also determined the myohemoglobin clearance rate and found it to be 55 to 60 per cent of that of creatinine. The difference can be explained on the basis of the molecular weight of the two hemoglobins. Myohemoglobin has a molecular weight one-fourth of that of hemoglobin, or approximately 17,000. Monke and Yuile<sup>69</sup> postulate that only 3 per cent of the glomerular pores are electrostatically large enough to allow the escape of the hemoglobin molecule. The uniformity of the process indicates that it is not a matter of glomerular injury.

The threshold for hemoglobin has been determined in human subjects a number of times. Sellards and Minot<sup>60</sup> showed that injection of hemoglobin solution into human subjects could be carried out without untoward reactions. They used as much as 33 c.c. of packed red blood cells hemolyzed with distilled water. Gilligan and co-workers<sup>43</sup> injected 1.3 to 16.4 Gm. of hemoglobin in the form of hemolyzed red blood cells into fifteen human subjects. When the plasma level was increased above 135 mg. per 100 c.c., hemoglobin appeared in the urine. When this level was once exceeded, hemoglobin continued to appear in the urine until the level dropped to 30 to 50 mg. per 100 cubic centimeters. They found that albumin was excreted in addition to hemoglobin, even when there was no pre-existing albuminuria, and continued to be excreted for a short time after hemoglobinuria ceased.

Ottenberg and Fox<sup>72</sup> injected 3.47 to 8.25 Gm. of hemoglobin as hemolyzed red blood cells into twenty subjects. They found that the renal threshold was quite variable and that five of thirteen subjects with plasma levels from 200 to 288 mg. per 100 c.c. did not have hemoglobinuria. When the plasma hemoglobin level did not exceed the threshold, the curve of disappearance of hemoglobin from the plasma was a straight line.

O'Shaughnessy and associates,<sup>72</sup> Duesberg,<sup>27</sup> and Fairley<sup>32-34</sup> also have injected hemoglobin solutions into human subjects. The largest dose injected into any patient was 50 Gm.,<sup>72</sup> and this amount produced some subjective symptoms but no renal injury. Hemoglobinuria lasted thirty hours. Other investigators also have noted rigors and some pain in the muscles and back from injections of hemoglobin. Duesberg<sup>27</sup> showed that the bilirubin concentration of the serum had increased thirty minutes after injection was completed and reached a peak a variable time later depending on the dose of hemoglobin and the patient's condition. Ottenberg and Fox<sup>72</sup> confirmed this observation.

The amount of hemoglobin excreted by the kidney after intravenous injection of a solution depends on the dose. Drabkin and associates' statement<sup>26</sup> that 9.5 per cent of the injected dose is excreted has been quoted widely, but it has been clearly demonstrated that the percentage varies with the dose injected unless oliguria or anuria are produced and that it may be as great as 33 per cent of the initial dose.<sup>43, 72, 96</sup> Likewise the duration of hemoglobinuria varies directly with the dose injected but also depends somewhat on the general condition of the patient or animal.

Reid<sup>77</sup> placed the kidney of a number of dogs in collodion plethysmographs and determined the effect of the injection of distilled water on the renal volume. There was a transient marked decrease in the volume of the kidney after the injection of 0.5 to 1.5 c.c. of water for each kilogram of body weight. The shrinkage lasted one or two minutes. He also injected laked dog blood made isotonic with sodium chloride. Five cubic centimeters of laked dog red blood cells caused the most marked decrease in renal volume, lasting up to twelve minutes. Anesthesia did not abolish the decrease in volume. A denervated kidney responded the same as the normal kidney. Mason and Mann<sup>67</sup> made direct observations on the frog kidney following the intravenous administration of a solution of pure hemoglobin and concluded that the decrease in volume of the kidney resulted from generalized contraction of the renal arterioles and arteries.

Hesse and Filatov<sup>48</sup> found that the injection of homologous hemoglobin solutions caused decrease in the renal volume lasting from several to twenty-five minutes, depending on the dose of hemoglobin. Heterologous blood caused a similar shrinkage of the kidney. Initially they found that complete denervation of the kidney prevented the shrinkage of the observed kidney. However, in later experiments by a group of investigators<sup>89</sup> from the same laboratory, it was clear that decerebration, transection of the cord, cutting the splanchnic nerves, and denervation of the kidney did not prevent the shrinkage; therefore, they concluded that the hemoglobin itself caused constriction of the renal vessels.

Hesse and Filatov<sup>48</sup> showed that transfusion of compatible blood promptly relieved the spasm of the renal vessels, that is, the volume and pulsations of the kidney returned quickly to normal. On the basis of this evidence they recommend the use of compatible blood transfusion as a treatment of hemolytic transfusion reaction. They believed that all the manifestations of hemolytic transfusion reactions could be explained on the basis of spasm of renal vessels, but later reports by members of the same group, Iljin and Mincev,<sup>53, 54</sup> conclude that some factor in addition to spasm accounts for renal damage.

Amberson and co-workers<sup>2</sup> were able to replace completely the blood of cats, dogs, and rabbits with a Ringer-Locke solution containing 12 to 14 Gm. of beef hemoglobin per 100 cubic centimeters. The animals were able to walk around the laboratory but they did not eat or drink and anuria developed. Some lived as long as thirty-six hours.

De Navasquez<sup>25</sup> injected hemoglobin solutions into rabbits and determined the iron content of the kidneys. He found that the animals excreting acid urine retained less iron and for a shorter time than those excreting alkaline urine. On the basis of this evidence he concluded that the rabbit was able to excrete hemoglobin better when the urine was acid. The dose of hemoglobin was relatively small, less than 1.0 Gm. per kilogram. Foy and associates<sup>40</sup> cite evidence that the mammalian kidney is better able to excrete solids when the urine is acid than when the urine is alkaline. This argument, however, does not take into account the matter of solubility of hemoglobin at various pH levels.

Maegraith and Havard<sup>65</sup> summarized mortality statistics of blackwater fever before the use of alkalis and after their use. In their summary of the series of cases reported by three different authors the mortality rate ranged from 19 to 25 per cent before the use of alkalis. In five different reports, but of a smaller number of patients receiving alkalis, the mortality rate ranged from 20 to 37 per cent. Maegraith and Havard concluded that alkali therapy has not benefited a group though it may have helped individual patients. They also pointed out the fact that patients with oliguria or anuria often continue to have an acid urine in spite of continuing alkali therapy. Anuria developed in some cases where the urine was alkaline immediately before the hemoglobinuria and continued to be alkaline after oliguria commenced. Foy and Kondi<sup>29</sup> likewise have observed that patients with alkaline urine develop anuria.

De Navasquez<sup>25</sup> acidified the urine of a patient with paroxysmal hemoglobinuria due to cold and then induced an attack by immersion of the arm in ice water. Hemoglobinuria lasted six hours, the highest plasma hemoglobin value was 688 mg. per 100 c.c., and an estimated 13.0 Gm. of hemoglobin were liberated. No renal insufficiency developed, and De Navasquez cites this as evidence that an acid urine is not a factor in renal damage resulting from hemoglobinemia. This, however, represents but a small amount of hemolysis, and it is probably incorrect to draw conclusions one way or the other from De Navasquez' experiment.

There are several considerations which are overlooked in most discussions of hemoglobinemic states, and it would seem that the most important of these is



the actual amount of hemoglobinemia present at the height of the reaction prior to the onset of oliguria or anuria. It is insufficient to determine the level of plasma hemoglobin a day or two after the onset of hemolysis, for it has been amply demonstrated that hemoglobin is rapidly removed from the blood stream in man and in animals.<sup>33, 34, 43, 72, 73</sup> Foy and Kondi<sup>38</sup> determined hemoglobin concentration of their patients with blackwater fever twelve to twenty-four hours after onset. Though they make a statement that the amount of hemolysis seems to be unimportant, their protocols indicate that the patients who died from anuria had the highest levels of plasma hemoglobin. Fairley and Bromfield<sup>31</sup> found that the highest hemoglobin concentrations occurred in their three fatal cases (3.6, 4.6, and 5.14 per cent of normal hemoglobin concentration in these cases).

The plasma level of hemoglobin was determined in four cases of hemolytic anemia due to sulfonamides by Fox and Ottenberg.<sup>37</sup> Twelve to forty-eight hours after the onset of hemolysis the total hemoglobin pigment levels ranged from 0.4 to 1.7 Gm. per 100 cubic centimeters. Two patients died from anemia and renal insufficiency. At autopsy on one patient dying three days after onset, the kidneys showed a large number of hemoglobin casts chiefly in the loops of Henle and distal tubules. Foy and associates<sup>41</sup> studied a patient dying from uremia as a result of an acute hemolytic anemia due to benzyl sulfanilamide. Quantitative determinations of the urine and plasma pigments were carried out, but no mention is made of the hemoglobin level in the plasma until the fifth day after jaundice was noted. At that time there were 291 mg. of methemalbumin (the pigment described by Fairley<sup>34</sup>) and 90 mg. of oxyhemoglobin per 100 c.c. but no methemoglobin.

The plasma hemoglobin levels in cases of paroxysmal nocturnal hemoglobinuria (Marchiafava-Micheli type) studied by Ham<sup>40</sup> and Hoffman and Kracke<sup>52</sup> ranged as high as 279 and 288 mg. hemoglobin per 100 c.c. of plasma. Ham reports one death from renal insufficiency but this patient had an acute pyelonephritis. Altshule and Gilligan<sup>3</sup> report a case of acute massive hemoglobinuria with jaundice and anemia. The nonprotein nitrogen rose to 90 mg. per 100 c.c. of blood during the course of the hemoglobinuria but returned to normal afterward. The plasma hemoglobin level was 500 mg. per 100 c.c. twenty-four hours after the onset, but it may well have been higher at some time earlier. Hemoglobinemia continued for eighteen days.

Recently, Shen and co-workers<sup>81</sup> studied eleven patients with hemoglobinuria out of forty with extensive second and third degree burns. Plasma or serum hemoglobin levels ranged from 65 to 215 mg. per 100 c.c. at an unspecified time after the burn. In four out of five patients with hemoglobinuria who lived five days or longer, azotemia of moderate severity occurred in spite of re-establishment of adequate urine flow with fixed low specific gravity of the urine. The kidneys of six patients were studied histologically and were consistent with hemoglobinuria. Olson and Necheles<sup>71</sup> studied burns experimentally and found that renal insufficiency was most likely to occur when the plasma hemoglobin level was highest. They found levels as high as 2,000 mg. per 100 cubic centimeters.

## EXPERIMENTAL METHODS

*Animal Experiments (Explantation of One Kidney).*—Explantation of the kidney has been carried out many times in the past. The first mention of this procedure is made by Allen in 1925.<sup>1</sup> Loesch<sup>2, 3</sup> explanted kidneys for the purpose of study of experimental hypertension. The renal pedicle was clamped intermittently and hypertension was produced. Biopsies were obtained by subjecting the dog to a surgical procedure. Entz and Huggins<sup>29</sup> explanted the kidney extracapsularly and were able to protect it sufficiently to prevent ulceration until a thick leathery capsule developed. Total kidney function as measured by the phenolsulfonphthalein test was unaffected by the procedure. Rhoads<sup>28</sup> carried out a somewhat more elaborate procedure in that the renal vein was also anchored subcutaneously so that repeated samples of renal vein blood were obtainable. Explantation of one kidney caused no change in the urea clearance. Removal of the other kidney caused no greater fall in urea clearance than if the remaining kidney had been in normal position. Gabriele<sup>12</sup> showed that the renal blood flow remained the same if the kidney were displaced outward and downward. However, if the renal pedicle were moved upward to form an acute angle with the great vessels, renal blood flow in that kidney decreased. Autopsies in the dogs used in the present experiments revealed an angle of 90 degrees or more with the great vessels and no kinking or stretching were found. Explantation of a kidney does not alter its blood flow appreciably.

Mongrel dogs weighing from 6.25 to 13.6 kilograms were chosen as the experimental animals. Each dog was anesthetized with sodium pentobarbital given intravenously. An oblique incision was made along the right costal margin beginning about 3 cm. from the midline dorsally. The muscle layers immediately caudal to the costal margin were split along the direction of their fibers. The peritoneum was incised and the kidney freed from its peritoneum and perinephric fat by blunt dissection. Care was taken not to dissect around the renal pedicle for the kidney could be mobilized adequately without interfering with the pedicle. The kidney was delivered through the opening in the muscle layers and rotated through an arc of about 75 degrees so that the upper pole was directed dorsally and slightly cephalad. The peritoneum and muscle layers were closed loosely around the pedicle using silk or linen suture material. Finally the skin was sutured using sulfathiazole powder along the incision. No dressings were applied. The skin sutures were removed on the sixth or seventh days. In several dogs there was a minor separation of the skin margins which healed satisfactorily in a short while. One dog suffered a wide separation of the skin margins on the fifth day directly exposing the kidney. The kidney became ulcerated and finally it was necessary to sacrifice the dog without using it for an experiment. This was the only failure out of eleven dogs operated upon. The postoperative tissue reaction usually subsided in two or three weeks. The kidney was mobile to a certain extent but often the skin was attached to it.

*Biopsy Technique.*—When the reaction to operation had completely subsided the area over the explanted kidney was shaved and prepared with Novak's antiseptic. One per cent procaine hydrochloride solution was used as a local anesthetic. A Silverman needle\* was used to obtain biopsies of the kidney. Tripoli and Faders<sup>34</sup> and Hoffbauer<sup>31</sup> have used this instrument for biopsy of the liver. The author is indebted to Dr. Hoffbauer for helpful suggestions regarding the use of the needle. The poles of the kidney were used for biopsy sites for the most part. The needle was directed in such a way as to avoid the hilus region. The core of kidney tissue removed was placed in 10 per cent formalin in physiologic saline solution. As soon as the needle was removed the kidney was compressed between two fingers for a period of three to five minutes; it was possible to prevent hematoma formation at the site of biopsy by this compression. About 80 per cent of attempted biopsies were successful in obtaining satisfactory tissue for sectioning. In earlier experiments biopsies were obtained prior to any injection, but in later experiments biopsies were obtained only after injection. Sixty satisfactory biopsies were made and as many as ten from a single kidney were obtained.

\*Vlm No. 14 made by the MacGregor Instrument Co., Needham, Mass.

The specimens were mounted in paraffin and stained with hematoxylin eosin stain. Some of the autopsy specimens were also stained for free iron using potassium ferrieyanide and hydrochloric acid.

Biopsies were not entirely innocuous in every instance. One animal (Dog 6) died as a result of abscesses in the lower pole of the kidney at the site of a biopsy, and another (Dog 11) had a small abscess at one pole of the kidney. Dog 9 had a hematoma in the lower pole of the kidney as a result of the operation or of the control biopsy, most likely the former. At autopsy most of the kidneys were pitted at the site of some biopsy but only very small areas were involved.

*Preparation of Hemoglobin and Hemolyzed Red Blood Cell Solutions.*—Blood was obtained from dogs which were to be sacrificed for various reasons but were not ill. The blood was collected in sterile containers with sodium citrate as an anticoagulant. The red blood cells were allowed to sediment for four or five days, the plasma was removed by aspiration, and the remaining cells were washed three times with sterile sodium chloride solution (The cells were not washed for Experiments 2 through 4). The washed cells then were stored in a deep freezing unit (the cells were laked immediately for experiment 7b). The night before contemplated use two volumes of sterile distilled water were added to the cells. After several hours an amount of sterile hypertonic sodium chloride solution was added to make the solution isotonic. The laked cells were then centrifuged for one hour and the supernatant solution was removed by aspiration. The process of laking the cells and making the solution was carried out in the twelve-hour period before the use of the solution which was stored in a refrigerator all the time except during centrifugation. Spectroscopic examination of each solution was carried out immediately before its administration, and oxyhemoglobin alone was observed in all of the solutions prepared by laking the blood cells as previously described. Cultures were obtained on four occasions and no bacterial contamination was found at any time. Sterile precautions were taken in making all of the solutions. The hemolyzed erythrocyte solutions were used in Dogs 2 to 9.

The method of Bing<sup>11</sup> was used to prepare crystalline dog hemoglobin for use in three experiments for Dogs 10 and 11. The blood was collected and treated as described to obtain red blood cells. One-fifth volume of toluene was added to each bottle of washed cells; the bottles were vigorously shaken for five minutes and then allowed to stay in the refrigerator for twenty-four hours. The mixture was then centrifuged and the toluene layer and a creamy layer, presumably the stroma and lipoid layer, were decanted. The crystalline deposit was washed three times with cold sterile distilled water and centrifuged each time. Finally the crystalline deposit was dissolved in sterile saline solution. The crystals were found to be relatively insoluble unless some sterile sodium bicarbonate solution was added. (The amounts will be specified in individual experiments.) Before injection in each instance the solution was centrifuged and the clear supernatant solution used for intravenous injection. Cultures of this solution were found to be sterile in each of two instances in which cultures were obtained. As will be seen, much larger amounts of hemoglobin were used than were used by Bing. Methemoglobin was not detected by spectroscopic examination in the first two solutions used but was detected in the solution used in Experiment 10b and in one solution which was discarded.

*Injection Experiments.*—Each dog was given either ammonium chloride or sodium bicarbonate by mouth or stomach tube before the injection. At first 10 Gm. of either sub-bicarbonate quite well and this method was entirely satisfactory in producing alkaline urine each time it was used. The ammonium chloride was not tolerated well. In most instances it caused vomiting and several dogs exhibited convulsive disturbances immediately after intubation. On these occasions experiments had to be postponed and the hemoglobin solutions discarded. Enteric-coated ammonium chloride tablets given orally in doses of 3 Gm. at approximately intervals of eighteen hours, twelve hours, and one hour before the experiment proved to be very satisfactory in producing an acid urine and were well tolerated. It is difficult to understand how as much as 120 Gm. of ammonium chloride could have been given to dogs in forty-eight hours as described by Bing<sup>11</sup> without causing severe disturbances.

The dogs were anesthetized with sodium pentobarbital given intravenously and catheterized leaving the catheter in place for eight to ten hours after completion of the injection of hemoglobin or hemolyzed blood solution. No. 8 French ureteral catheters were used for male dogs. Blood was obtained for hematocrit determination after which the solution was injected at a rate of about 20 c.c. per minute into a forepaw vein. Subsequently, blood specimens for plasma examination and hematocrit were obtained by venepuncture or femoral artery puncture immediately after completion of injection and at intervals during the first eight hours. Isotonic sodium citrate was used as an anticoagulant in the first four experiments, but a correction for the volume of citrate solution is necessary since an appreciable dilution occurs when one volume of solution to nine volumes of blood are used. For the rest of the experiments one drop of 25 per cent sodium citrate solution per 5 c.c. of blood was used and no correction for dilution was made. Artificial hemolysis is not induced by the 25 per cent sodium citrate when the blood is otherwise handled with care.

Urine was collected at variable intervals depending on the rate of urine flow. An attempt was made to collect separately urine of grossly comparable concentration. After removal of the catheter all the urine was collected by the use of metabolism cages. Freshly voided specimens were collected when possible during the first few days after the injection.

*Chemical Procedures and Other Methods.*—The concentration of hemoglobin in the solution, the plasma, and the urine was determined by the pyridine hemochromogen method described previously.<sup>35</sup> The urine hemoglobin concentrations were determined directly, that is, without extraction with ether, for the concentrations were great. It was found that no error resulted by using the method described for plasma. Both urine and plasma had such great concentrations of hemoglobin that it was necessary to use 0.02 to 0.10 c.c. samples initially and from 0.1 to 0.5 c.c. later. It is necessary to use the reagent grade of pyridine in order to get uniform results. The method is as follows: The citrated blood is centrifuged as soon as it is obtained and the plasma is withdrawn. Plasma samples (0.02 to 0.05 c.c. or more up to 0.5 c.c.) are placed in Evelyn colorimeter tubes. The plasma is diluted with dilute ammonium hydroxide to 7.5 c.c. and finally to 10 c.c. with 0.5 c.c. of pyridine and 2.0 c.c. of freshly prepared 0.2 per cent sodium hydrosulfite. A duplicate tube for center setting determinations is set up in the same manner but with 1.0 c.c. less ammonium hydroxide, thus allowing for 1.0 c.c. of 3 per cent hydrogen peroxide. Readings are obtained in five minutes. The following formula is used:

$$\text{Concentration} = \frac{1000 \times L}{K \times V} \text{ mg. hemoglobin per 100 c.c. plasma}$$

L - (2 - log G) = "L" value

G - galvanometer readings

V = c.c. of plasma used

K = 1.843.

Hematocrit determinations were made using dry mixed ammonium and potassium oxalate as anticoagulant and the Wintrobe hematocrit tube. Unfortunately, several control specimens clotted so that some of the experiments had to be excluded from certain calculations involving hemodilution, etc. Blood specimens were usually obtained at twenty-four hour intervals for the duration of hemoglobinemia and at variable intervals for the determination of the blood urea nitrogen, depending on the circumstances of each experiment. The blood urea nitrogen was determined by the direct nesslerization of a Folin-Wu filtrate of blood incubated with urease.<sup>75</sup> The method has been adapted for use with the Evelyn colorimeter.

Microscopic examination of urine sediment was carried out at intervals as seen in the tables of each experiment. Only fresh urine specimens were examined.

Spectroscopic examinations were carried out using a Zeiss band spectroscope. Methemoglobin concentrations in Experiment 10b were carried out by the method of Evelyn and Malloy.<sup>50</sup>

The pH of the urine was determined by the use of a Coleman No. 3 electrometer. When the reaction is recorded as alkaline or acid, alkacid ribbon\* was used. The pH was determined in two hours or less after collection directly from the dog.

\*Fisher Scientific Co., Pittsburgh, Pa.

## EXPERIMENTAL OBSERVATIONS

Nineteen experiments on ten dogs were done. Serial biopsies were obtained in eleven experiments and were satisfactory for pathologic examination. The earliest biopsies were obtained four to eight hours after injection of hemoglobin solutions in Experiments 3, 4, 5a, 6, 7a, 8, 9, and 10b and twenty-four hours afterward in Experiments 7b, 10a, and 11.

Data regarding the volume, quantity of hemoglobin, plasma hemoglobin levels, hemodilution as measured by hematocrit changes, duration and extent of hemoglobinuria, pH of the urine during the first twenty-four hours, blood urea nitrogen levels, presence of casts are recorded in Tables I and II. The data regarding the first four experiments are not included because of the relatively small quantities of hemoglobin injected, but some of these experiments are represented on the graphs listed as Experiment 2a to 2d. Two dogs died shortly after the injection was completed (Experiments 2g and 5b).

Dogs 5a, 6, 7a, 7b, and 10a recovered promptly from the effect of the infusions and anesthesia. Dogs 5a, 6, and 7a had acid urine and Dogs 7b and 10a had alkaline urine. Dog 6 developed an infection (proved to be renal abscesses at autopsy) and became progressively more ill after the fourth day. None of the above dogs developed renal insufficiency. Dogs in Experiments 3, 4, 8, 9, 10b, and 11 developed renal insufficiency at least temporarily or died from uremia. Dogs 3, 4, 8, and 10b had alkaline urine and Dogs 9 and 11 had acid urine. Dog 3 was seriously ill for about three weeks; then it recovered partially but contracted a respiratory infection and died from pneumonia on the forty-sixth day. Dog 8 was ill from the time of injection and contracted a respiratory infection on the fourth day but responded to sulfadiazine therapy and recovered. Dog 4 was ill for three or four days but recovered completely. Dogs 9 and 11 became progressively more ill and had twitchings and convulsions terminally, vomited frequently, and died from uremia. Dog 10b died approximately thirty-six hours after injection; the immediate cause of death was not determined but there was extensive renal damage at autopsy.

One animal (Dog 5b) died three hours after the injection, either from shock or from cardiac irregularity which was noted just before death. There was a moderate amount of free peritoneal fluid which contained 2.5 Gm. of hemoglobin per 100 cubic centimeters. In the other experiments the spleen and liver often were engorged at the time of injection and the veins were greatly distended. In Experiment 2g the dog died about eight hours after completion of the injection. The spleen was large and the peritoneal cavity contained hemoglobin-stained fluid. All the organs had hemorrhagic areas throughout their substance. The lumen of the intestines contained a considerable quantity of blood.

Unfortunately blood pressure determinations were not obtained. From observation of the pulse volume there probably was some lowering of the blood pressure in the first eight-hour period. It is significant that at no time did anuria occur; in other words, the blood pressure did not decrease enough to cause complete failure of excretion of urine.

TABLE I. SUMMARY OF DATA REGARDING DOSE OF HEMOGLOBIN, PLASMA LEVELS OF HEMOGLOBIN, HEMODILUTION, AND HEMOGLOBIN EXCRETION

DOG	WEIGHT (KG.)	HEMOGLOBIN SOLUTION			PLASMA HEMOGLOBIN (GM./100 C.C.) IN HOURS AFTER INJECTION OF HEMOGLOBIN SOLUTION								HEMODILUTION† (%)	EXCRETION (%)	HOURS OF HEMOGLO- BINURIA	REACTION OF URINE
		VOLUME (C.C.)	AMOUNT (GM.)	(GM./ KG.)	*											
					0	1/2	1	2	4	*	*	*				
2e	13.5	850	32.1	2.38	2.60	2.39	1.96	1.74	1.49	.82(10)	.16(31)	-	28	31	Alkaline	
2f	13.5	850	40.0	2.96	3.43	3.29	3.29	2.88	1.94(8)	.24(28)	.152	152	35	32	Acid	
3	12.7	900	68.8	5.42	4.70	4.18	4.15		2.41(7)	1.30(24)	.32(72)	148	18	72+	Alkaline	
4	12.7	700	54.0	4.25	3.75		2.91	2.34	1.37(8)	1.10(24)	.13(72)	180	36	72	Alkaline	
5a	8.18	575	36.9	4.50	3.18	2.96	2.74		1.24(10)	.73(27)	.33(48)	170	35	48	Acid	
6	6.25	525	30.2	4.80	3.46		2.80		2.02	1.77(8)	.51(24)	156	38	48+	Acid	
7a	7.9	590	41.0	5.20	3.12	2.94	2.66		2.40	1.57(8)	.45(24)	137	38	48	Acid	
7b	11.3	950	52.0	4.60	3.35	2.89	2.81		2.09	1.81(8)	.63(24)	137	30	48+	Alkaline	
8	7.95	700	45.5	5.74	3.32		2.94			2.18(8)	1.41(27)	143	31	72	Alkaline	
9	9.1	725	51.8	5.70	4.72				3.62	3.00(8)	.47(72)	-	27	72+	Acid	
10a†	6.45	950	39.8	6.15	3.38		3.04			2.48(8)	.67(24)	160	42	72	Alkaline	
10b†	8.1	450	48.4	5.95	5.63	5.35		4.89		3.83(8)	1.98(26)	192	24	36	Alkaline	
11†	8.18	975	43.8	5.36	3.78	3.55	3.46	(3)		2.06(8)	1.09(26)	176	37	Died	Acid	

\*The time in hours is indicated by the number in parentheses.

†Hemodilution is an expression of the ratio of initial hematocrit to the lowest hematocrit after injection.

‡Solutions of crystalline hemoglobin were used in these three experiments.

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Nineteen experiments on ten dogs were done. Serial biopsies were obtained in eleven experiments and were satisfactory for pathologic examination. The earliest biopsies were obtained four to eight hours after injection of hemoglobin solutions in Experiments 3, 4, 5a, 6, 7a, 8, 9, and 10b and twenty-four hours afterward in Experiments 7b, 10a, and 11.

Data regarding the volume, quantity of hemoglobin, plasma hemoglobin levels, hemodilution as measured by hematocrit changes, duration and extent of hemoglobinuria, pH of the urine during the first twenty-four hours, blood urea nitrogen levels, presence of casts are recorded in Tables I and II. The data regarding the first four experiments are not included because of the relatively small quantities of hemoglobin injected, but some of these experiments are represented on the graphs listed as Experiment 2a to 2d. Two dogs died shortly after the injection was completed (Experiments 2g and 5b).

Dogs 5a, 6, 7a, 7b, and 10a recovered promptly from the effect of the infusions and anesthesia. Dogs 5a, 6, and 7a had acid urine and Dogs 7b and 10a had alkaline urine. Dog 6 developed an infection (proved to be renal abscesses at autopsy) and became progressively more ill after the fourth day. None of the above dogs developed renal insufficiency. Dogs in Experiments 3, 4, 8, 9, 10b, and 11 developed renal insufficiency at least temporarily or died from uremia. Dogs 3, 4, 8, and 10b had alkaline urine and Dogs 9 and 11 had acid urine. Dog 3 was seriously ill for about three weeks; then it recovered partially but contracted a respiratory infection and died from pneumonia on the forty-sixth day. Dog 8 was ill from the time of injection and contracted a respiratory infection on the fourth day but responded to sulfadiazine therapy and recovered. Dog 4 was ill for three or four days but recovered completely. Dogs 9 and 11 became progressively more ill and had twitchings and convulsions terminally, vomited frequently, and died from uremia. Dog 10b died approximately thirty-six hours after injection; the immediate cause of death was not determined but there was extensive renal damage at autopsy.

One animal (Dog 5b) died three hours after the injection, either from shock or from cardiac irregularity which was noted just before death. There was a moderate amount of free peritoneal fluid which contained 2.5 Gm. of hemoglobin per 100 cubic centimeters. In the other experiments the spleen and liver often were engorged at the time of injection and the veins were greatly distended. In Experiment 2g the dog died about eight hours after completion of the injection. The spleen was large and the peritoneal cavity contained hemoglobin-stained fluid. All the organs had hemorrhagic areas throughout their substance. The lumen of the intestines contained a considerable quantity of blood.

Unfortunately blood pressure determinations were not obtained. From observation of the pulse volume there probably was some lowering of the blood pressure in the first eight-hour period. It is significant that at no time did anuria occur; in other words, the blood pressure did not decrease enough to cause complete failure of excretion of urine.

TABLE I. SUMMARY OF DATA REGARDING DOSE OF HEMOGLOBIN, PLASMA LEVELS OF HEMOGLOBIN, HEMODILUTION, AND HEMOGLOBIN EXCRETION

NO.	WEIGHT (KG.)	HEMOGLOBIN SOLUTION		PLASMA HEMOGLOBIN (GM./100 G.C.) IN HOURS AFTER INJECTION OF HEMOGLOBIN SOLUTION							HEMODILUTION† (%)	EXCRETION (%)	HOURS OF HEMOGLOBINURIA	REACTION OF URINE
		VOLUME (C.C.)	AMOUNT (GM.)	(GM./ KG.)	0	1/2	1	2	4	*	*			
2c	13.5	850	32.1	2.38	2.00	2.39	1.96	1.74	1.49	.82(10)	.16(31)	-	28	31 Alkaline
2f	13.5	850	40.0	2.96	3.43	3.29	3.29	2.88		1.94(8)	.24(28)	152	35	32 Acid
3	12.7	900	68.8	5.42	4.70	4.18	4.15			2.41(7)	1.30(24)	148	18	72+ Alkaline
4	12.7	700	54.0	4.25	3.75		2.91	2.34		1.37(8)	1.10(24)	180	36	72 Alkaline
5a	8.18	575	36.9	4.50	3.18	2.96	2.74			1.24(10)	.73(27)	170	35	48 Acid
6	6.25	525	30.2	4.80	3.46		2.80		2.02	1.77(8)	.51(24)	156	38	48+ Acid
7a	7.9	590	41.0	5.20	3.12	2.94	2.66		2.40	1.57(8)	.45(24)	137	38	48 Acid
7b	11.3	950	52.0	4.60	3.35	2.89	2.81		2.09	1.81(8)	.63(24)	-	30	48+ Alkaline
8	7.95	700	45.5	5.74	3.32		2.94			2.18(8)	1.41(27)	143	31	72 Alkaline
9	9.1	725	51.8	5.70	4.72			4.12	3.62	3.00(8)	2.45(26)	-	27	72+ Acid
10a†	6.45	950	39.8	6.15	3.38		3.04			2.48(8)	.67(24)	160	42	72 Alkaline
10b†	8.1	450	48.4	5.95	5.63	5.35		4.89		3.83(8)	1.98(26)	192	24	36 Alkaline
11†	8.18	975	43.8	5.30	3.78	3.55	3.46	(3)		2.06(8)	1.09(26)	176	37	72 Acid

\*The time in hours is indicated by the number in parentheses.

†Hemodilution is an expression of the ratio of initial hematocrit to the lowest hematocrit after injection.

‡Solutions of crystalline hemoglobin were used in these three experiments.



TABLE II. SUMMARY OF DATA REGARDING BLOOD UREA NITROGENS, URINE PH, AND URINALYSIS

DOG	DOSE OF HEMO- GLOBIN (GM./ KG.)	MEDICATION IN GRAMS		BLOOD UREA NITROGEN (MG./100 C.C.)							URINE VOLUME					
				(MG./100 C.C.)							RANGE OF PH FIRST DAY	CASTS	TIME OF APPEAR- ANCE OF METHEM- OGLOBIN (HR.)	FIRST DAY (C.C.)	RANGE OF VOLUME FIRST WEEK	EXCRE- TION (% OF TOTAL DOSE)
		Na HCO <sub>3</sub>	NH <sub>4</sub> Cl	TWO DAYS	THREE DAYS	FOUR DAYS	SEVEN DAYS	*								
2e	2.38			11					8.1 to 8.2	0		766		27.6	31	
2f	2.9			15					5.9 to 6.5	+	2	1,352		35	32	
3	5.42	10		72	-	92	85	53(46)	7.6 to 8.5	++	10	901	975 to 500	17.5	72+	+
4	4.25	10		40		27	22	18(16)	7.6 to 8.35	+	9	1,040	1,050 to 100	36	72	+
5a	4.5			12		11			6.7 to 7.1	+	3	404	200 to 700	35	48	0
6	4.8			22		53	11		6.0 to 6.7	++	2	450	150 to 1,100	38	48+	0
7a	5.2				15		17		5.8 to 6.8	++	2	1,283	150 to 1,325	37.8	48	0
7b	4.6	10		6	9	18			7.5 to 8.1	+	5	1,413	200 to 700	29.8	48+	0
8	5.74	11		39	46		31	32(16) 9(26)	7.7 to 8.4	++	8	785	400 to 1,000	31	72	Trace
9	5.7			120	200	300	300		5.5 to 6.6	++	1	633	150 to 872	27	72+	+
10a†	6.15	10		17		13			8.1 to 8.4	Occa- sional	10	1,090	250 to 440	42.5	72	0
10b†	5.95	5		56(1)					7.8 to 8.4	+	1	715	-	24	36	
11†	5.36			49	155	190	278(0)		5.5 to 7.0	++	1	755	350 to 1,100	37	72	Trace

\*The time in days is indicated in the parentheses.

†The volumes may not be accurate for the dogs with renal insufficiency after the third or fourth day, since these dogs vomited and the vomitus would be collected just as voided urine would be.

‡Solutions of crystalline hemoglobin were used in these experiments.

An attempt was made to determine the size of the kidney by palpation during the immediate postinjection period. In no instance did the kidney seem to shrink in size, but in several dogs it seemed to enlarge slightly. For several hours following injection the consistency was uniformly firmer than before. External measurements by means of a ruler confirmed the impression that shrinkage did not occur. Of course, there may have been some change in volume which could have been determined only by plethysmography.

From the data in Table II it is evident that methemoglobin appeared in the urine much earlier when the urine was acid than when it was alkaline. In all except Experiment 10b the hemoglobin solution contained no methemoglobin or less than is detectable by spectroscopic examination. The solution used in 10b contained 13 per cent methemoglobin and the rest was oxyhemoglobin.

The results of the renal biopsies can be summarized for Experiments 5a and 5b, 6, 7a and 7b and 10a by stating that little or no renal damage could be detected at any time after injection. Rarely casts were found in some of the tubules during the period of hemoglobinuria and were fairly numerous in Experiment 7a. (Sections are normal otherwise so they will not be illustrated.) The convoluted tubule cells contained many granules of pigment which had the staining qualities of hemosiderin. A few of these granules were found in the kidney as long as 112 days after injection (Dog 4). Hemosiderin granules were numerous in the reticulo-endothelial cells of the spleen and liver, too. The explanted kidney of Dog 6 was partially destroyed by multiple abscesses, but the rest of the kidney and the normally placed kidney were entirely normal at autopsy. The same fundamental structural changes were present in the normally placed kidney as in the explanted one at autopsy in every dog, except when an abscess (Dog 6 and 11) or hematoma (Dog 9) were found in the explanted kidney.

Casts appeared in the urine in abundance in practically every experiment. The casts were brown and granular in appearance. Although quantitative determinations were not made, it was apparent that the number of casts was greater in some experiments when the urine was acid than in any when it was alkaline. The casts were not identified chemically but very likely were derived from hemoglobin because of their brown-red color and their occurrence immediately after hemoglobinuria started. The casts in the tubules of the kidney did not stain with ferrieyanide so they did not contain any free iron. Many of the casts in the tubules had a crystalline structure. The casts stained uniformly with the eosin stain and had a color similar to red blood cells stained with eosin.

The pathologic findings in dogs with renal damage were more interesting. The urine was alkaline during the first twenty-four hours in Experiments 3, 4, 8, and 10b and acid in Experiments 9 and 11. An attempt will be made to illustrate early damage, its progress, and finally partial or complete healing. Space does not permit inclusion of description of all of the sections obtained; therefore, only the most characteristic findings will be described.

Four hours after the injection in Dog 3 there was slight vacuolization of the epithelial cells in the proximal and distal convoluted tubules and there were

Fig. 1.

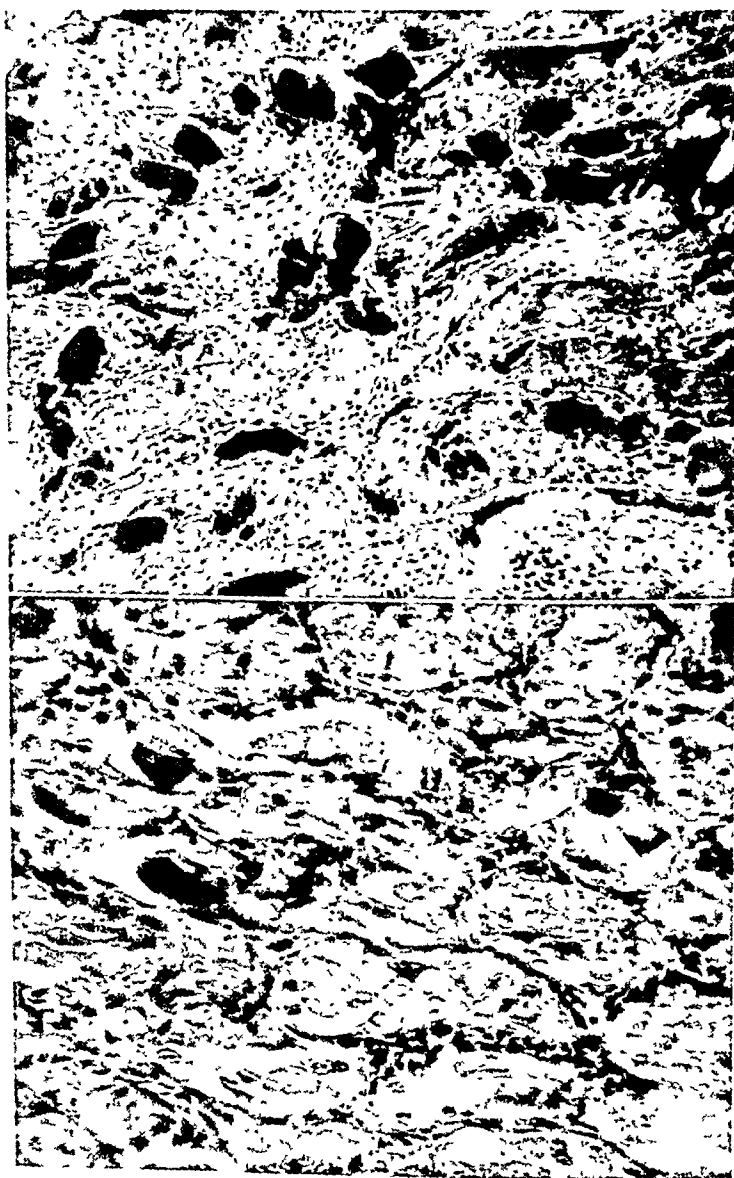


Fig. 2.

Fig. 1.—Kidney, Dog 3; alkaline urine; biopsy obtained one day after injection of hemoglobin solution. Numerous casts are evident and there are early degenerative changes in the tubular epithelium ( $\times 150$ ).

Fig. 2.—Kidney, Dog 3, biopsy obtained five days after injection of hemoglobin solution. There are very extensive degenerative changes in the tubule cells. Other portions of the same section have many hemoglobin casts in the tubules ( $\times 300$ ).

Fig. 3.

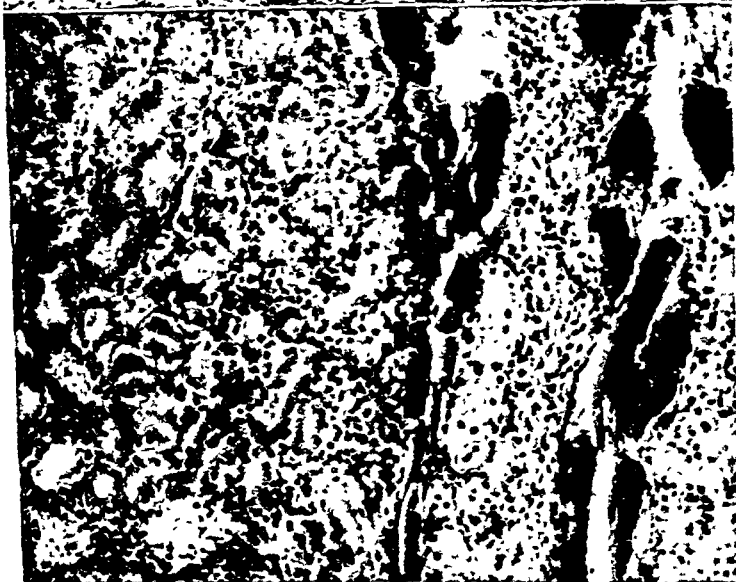


Fig. 4.

Fig. 3.—Kidney, Dog 3; biopsy obtained seventeen days after injection of hemoglobin solution. The epithelium of the convoluted tubules shows many evidences of healing as well as persisting degenerative changes in other areas (X150).

Fig. 4.—Kidney, Dog 3; autopsy forty-six days after injection of hemoglobin solution. There are narrow wedges of normal cortex interposed between areas of tubular atrophy. The convoluted tubule cells in the atrophic areas have many hemosiderin granules (when stained with a ferricyanide stain). The lumina of the atrophic tubules are narrow but patent. The photomicrograph depicts an area of healed cortex adjacent to one atrophy with large hemoglobin casts (X150).

many hemoglobin casts in the loops of Henle and of the distal convoluted tubules. The cytoplasm of the cells of the proximal convoluted tubules had red staining globules. Twenty-four hours after the injection the lumina of the majority of loops of Henle and Distal convoluted tubules were full of hemoglobin casts (Fig. 1). There was swelling of the cells of some proximal convoluted tubules and the nuclei were pyknotic. At forty-eight hours the tubular epithelium showed further degeneration. At five days extreme vacuolization of the cells and some disorganization of the tubules in both the cortex and cortico-medullary portion had appeared (Fig. 2). The number of casts in the cortico-medullary portion (not shown in the photomicrograph) was as numerous as on the first day. At ten days there was evidence of beginning regeneration of tubule cells. At seventeen days most of the casts were in the corticomedullary portion. In the cortical portion as shown in Fig. 3, some of the tubules were lined by flat or low cuboidal cells and apparently were undergoing atrophy, but others were regenerating and had nearly normal appearance.



Fig. 5.—Kidney, Dog 8; alkaline urine; autopsy thirty-five days after injection of hemoglobin solution. Most of the cortex appears to be normal, but there are wedge-shaped areas of atrophy in which the tubules are shrunk or dilated and there are hemoglobin casts in the medullary tubules ( $\times 205$ ).

At autopsy on the forty-sixth day the surfaces of the kidneys were finely pitted. On section it was evident that the pits were represented by radial streaks of atrophy. In an unstained section the medulla had brownish-red radial streaking. There were narrow wedges of normal cortex interspersed with areas of atrophy and casts. The lumina of the atrophic tubules were narrow but patent. Fig. 4 depicted an area of healed cortex adjacent to one of atrophy with large hemoglobin casts in the lumina of the atrophic tubules (forty-six days).

Fig. 6.

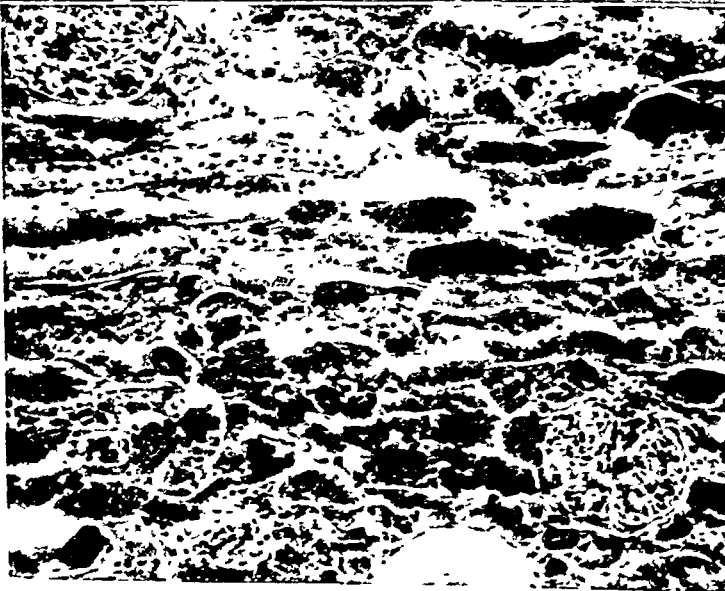
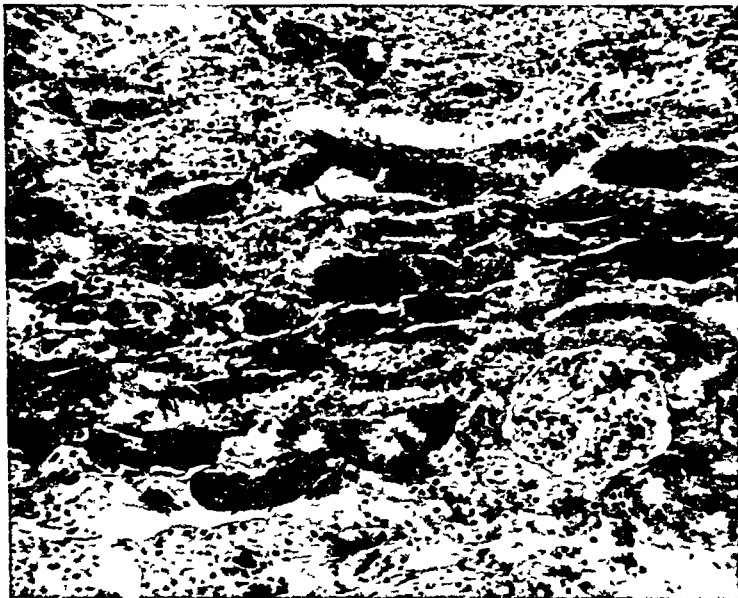


Fig. 7.

Fig. 6.—Kidney, Dog 4; alkaline urine; biopsy obtained five hours after injection of hemoglobin solution. A large number of hemoglobin casts are present in the loops of Henle and in the distal convoluted tubules. Some casts have a crystalline appearance, but others appear amorphous ( $\times 150$ ).

Fig. 7.—Kidney, Dog 10; alkaline urine; biopsy obtained eight hours after injection of solution of hemoglobin crystals. There are numerous casts throughout the cortex and early degenerative changes ( $\times 150$ ).

The findings in Dog 8 were similar qualitatively. The number of casts and the degenerative changes were less marked and the kidneys at autopsy showed much less permanent damage and casts than in Dog 3. Some of the atrophic tubules were dilated (Fig. 5). The renal function had returned practically to normal as evidenced by concentrating ability of the kidney. It is entirely possible that further repair of the anatomic damage would have occurred if the dog had been permitted to live.

The changes in Dog 4 were just as severe in the early stages as in Dog 8, but the progress toward complete healing was rapid after the eleventh day. Some pigment casts and atrophic tubules were evident in biopsies obtained at fifty-one and eighty-three days. At autopsy the kidneys were entirely normal with the exception of rare pigment casts and atrophic tubules. Fig. 6 from a biopsy obtained five hours after the hemoglobin injection revealed the very early appearance of casts in the tubules in spite of the continuous alkalinity of the urine. Experiments 4 and 8 illustrate the power of the kidney to regenerate after severe tubular damage.

In Experiment 10b the dog was given a solution made from 48.4 Gm. of crystalline hemoglobin. Thirteen per cent of the hemoglobin in this solution was methemoglobin. The pulse rate diminished to a low of 44 beats per minute six hours after the injection, and the pulse was weak. Twenty-four hours afterward the dog was listless and weak, but the pulse rate was 100 per minute and the pulse was strong. The dog was found dead forty-four hours after injection. Autopsy was not performed until fifty-two hours, or at least twelve hours after death. Unfortunately, considerable autolysis had occurred by this time. A biopsy (Fig. 7) obtained at eight hours revealed very many casts throughout the cortex. At autopsy the casts were more numerous. The tubule cells were badly degenerated (at least some of this represents post-mortem autolysis).

The results of Experiments 9 and 11 are so much alike that a description of the findings in Experiment 11 will suffice. Part of the explanted kidney in Dog 9 was replaced by an old hematoma. The hematoma probably was the result of trauma at the time of explantation, for there was troublesome bleeding from an artery over the lower pole. The hematoma may have been due to the initial biopsy.

In Experiment 9 hemolyzed red cells were used, but in Experiment 11 a solution made from crystalline hemoglobin was used. Dog 9 died on the seventh day and Dog 11 was killed on the tenth day, for it was obviously moribund. Each dog vomited after the third or fourth day but not before.

In Experiment 11, at twenty-four hours, there were many casts throughout the cortex and corticomedullary portion occluding the lumina of most of the loops of Henle and the distal segment. There were degenerative changes in the tubules not containing casts (Fig. 8). At three days degenerative changes were marked with desquamation of tubular epithelium, poor staining, and vacuolization of the cytoplasm and pyknosis of nuclei. Casts were very numerous. At six days the cells of the convoluted tubules were severely injured with more marked changes than noted at three days. At autopsy (ten days, Fig. 9) there

Fig. 8.

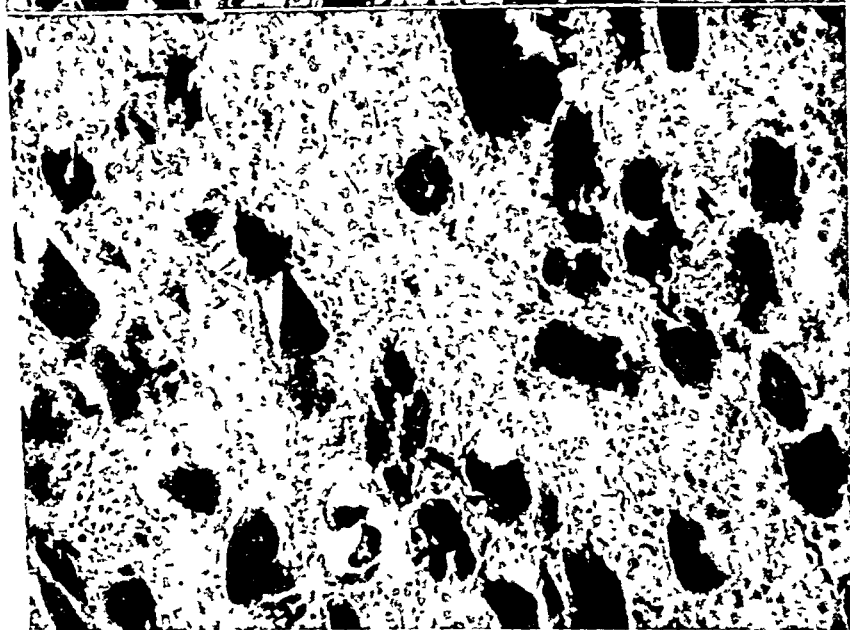
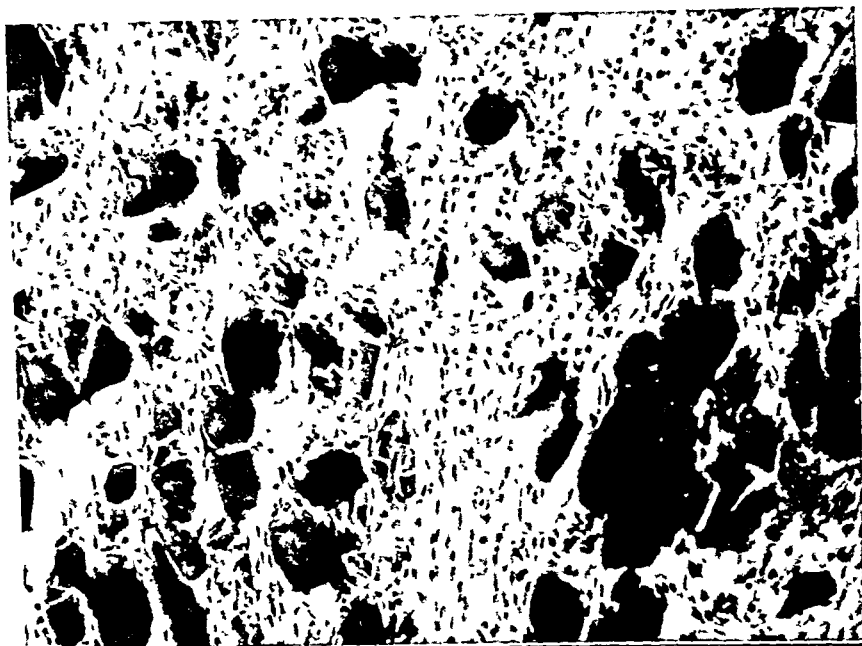


Fig. 9.

Fig. 8—Kidney, Dog 11; acid urine; biopsy obtained one day after injection of a solution of hemoglobin crystals. The number of hemoglobin casts is the greatest of any of the experiments. There are degenerative changes in the tubules in the cortex ( $\times 265$ ).

Fig. 9—Kidney, Dog 11; autopsy ten days after injection of solution of hemoglobin crystals. The number of casts remains as large in the medulla and corticomedullary portions but is less in the cortex. Cortical tubules show degenerative changes progressing to atrophy of some and dilatation of others ( $\times 265$ ).



were many casts in the corticomedullary portion. In the cortex there was evidence of atrophy of some tubules, dilatation of others, and continued degeneration as previously noted. There was evidence of healing of some of the degenerated tubule cells. The explanted kidney had a small abscess at the lower pole, but the rest of the pathologic findings were identical in the two kidneys.

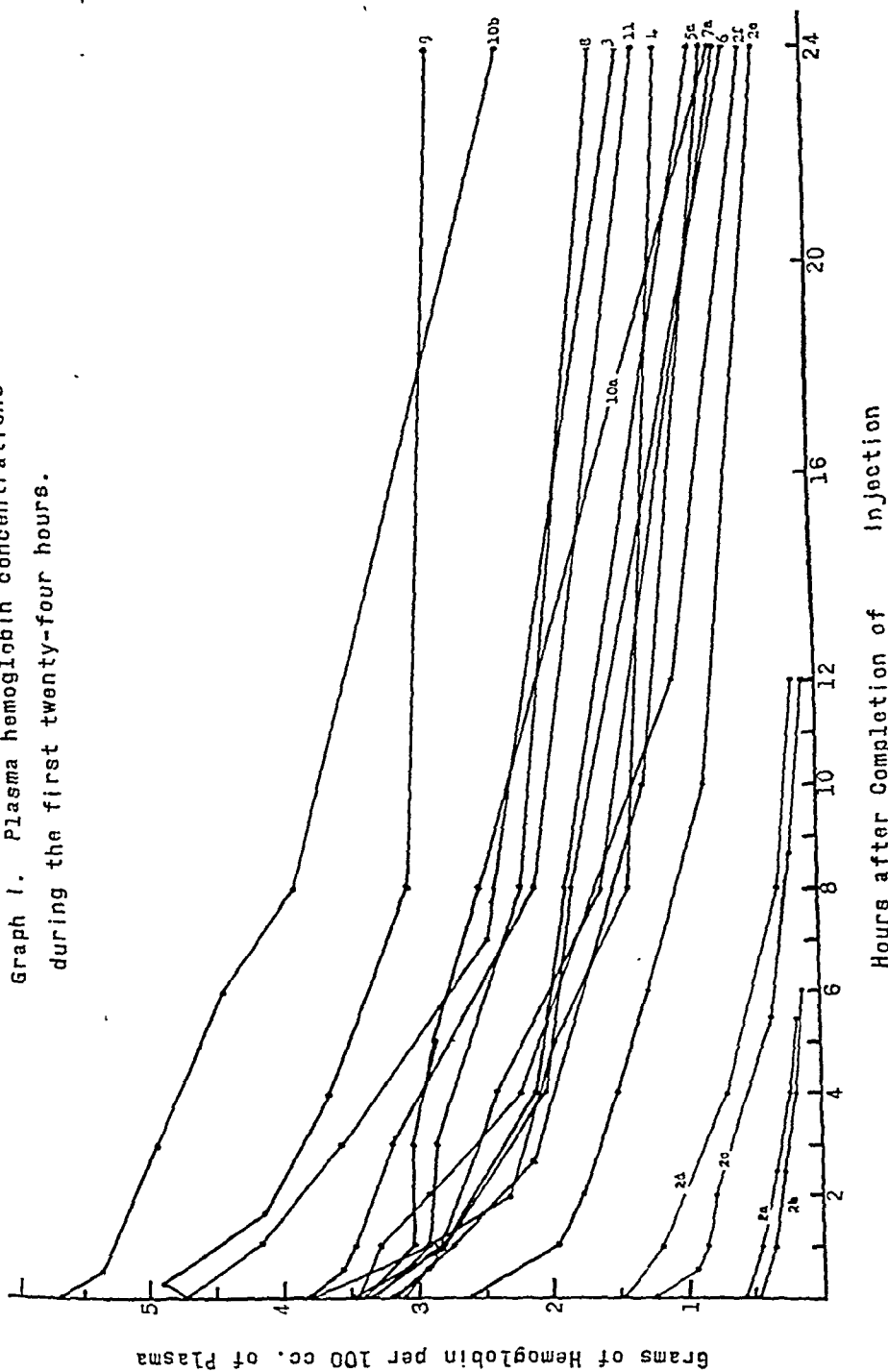
Microscopic examination of the urine was carried out at frequent intervals as indicated in Tables I and II. Casts described, unless otherwise specified, were large, dark brown, and granular. Some casts had square ends but most of them had a shape similar to any tubular casts. In Dog 11 casts appeared in the very first urine obtained after injection of hemoglobin but also in the urine of most dogs very soon after hemoglobinuria started. Because of catheterization the significance of hematuria is questionable.

In Dogs 3, 4, 8, 9, 10a, and 11 retention of urea nitrogen, excretion of urine of low fixed specific gravity, albuminuria, and in three dogs, death from uremia are interpreted as evidences of renal insufficiency. There probably was an increased rate of protein metabolism in these dogs. When one subtracts the amount excreted, the dose of the administered hemoglobin did not constitute more than a large protein meal which any dog can eat in one day. It is doubtful that vomiting and dehydration had any significant role in these cases until development of frank renal insufficiency and uremia. Dogs 9 and 11 did not begin to vomit until uremia was well established. Dog 4 developed only transient renal insufficiency and probably could be classed with the other group, but it is classed in the group with renal insufficiency because of the histologic manifestation of moderate characteristic renal damage. No dogs developed anuria, but the dogs with renal damage continued to excrete rather large volumes of low specific gravity urine. One determination of the ratio of the urine urea nitrogen to blood urea nitrogen was made. It was found to be 455/155 on the fifth day in Experiment 11. This is further evidence of failure to concentrate urinary solids. Other workers<sup>16, 31, 81</sup> have observed the development of uremia in spite of excretion of normal or even large volumes of urine.

Graph I depicts the disappearance of hemoglobin from the plasma as evidenced by the plasma hemoglobin levels during the first twenty-four hours. It may be noted that there is a rather sharp decline in levels during the first four hours, but after that there is a more gradual and uniform decline. Hemoglobinemia continued for forty-eight hours or longer in all of the cases except in the experiments on Dog 2. (See Tables I and II for these details.) The duration of hemoglobinemia is directly related to the dose of hemoglobin administered.

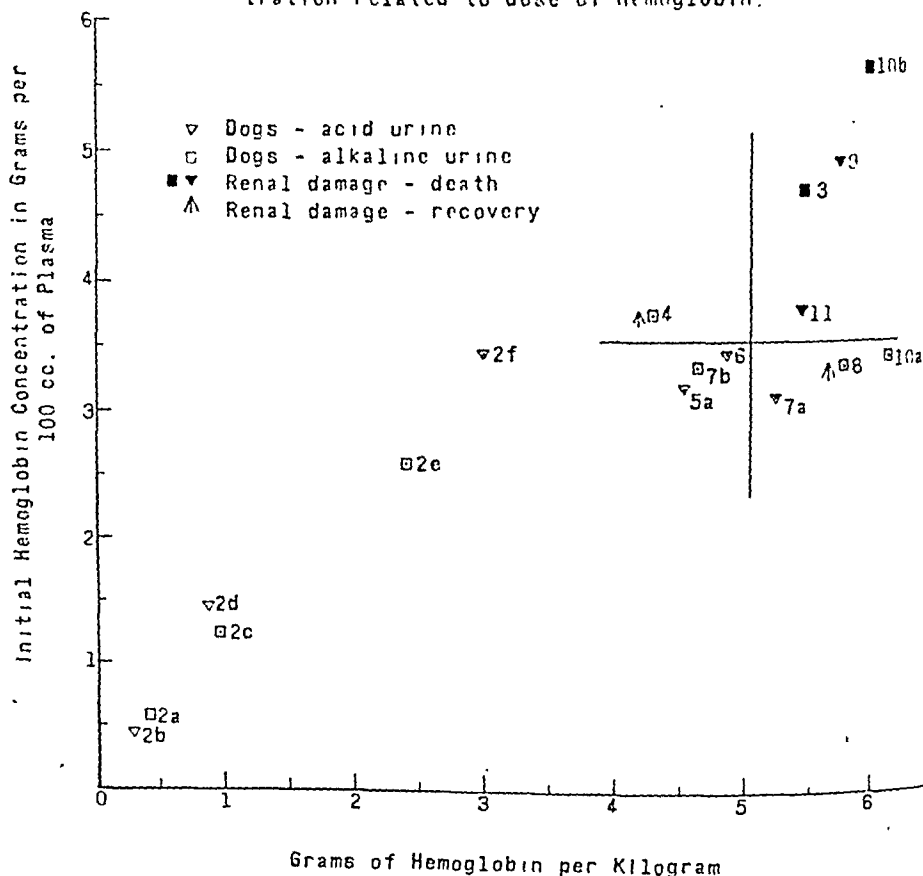
There is a relationship between the initial plasma hemoglobin level and the dose of hemoglobin, but as illustrated in Graph II there are individual variations which cannot be readily explained on the basis of differences in hemodilution. One factor of importance is the concentration of the administered solution. In every experiment (except Experiment 11) in which the initial plasma hemoglobin level was above 3.5 Gm. per 100 c.c., the concentration of the solution administered was greater than 7.0 Gm. per 100 cubic centimeters. Dog 10 is a good example of this effect, for with comparable doses of hemoglobin the initial

Graph I. Plasma hemoglobin concentrations during the first twenty-four hours.



plasma level was 3.38 when the concentration of the solution was 4.10 Gm. per 100 c.c. and 5.63 Gm. per 100 c.c. when the concentration of the solution was 10.74 Gm. per 100 cubic centimeters. Of course, the factor of concentration is affected by the time necessary for administration and also by the total dose administered, so the effect of concentration probably is a combination of factors. Nevertheless, it is desirable to administer as concentrated a solution as possible in order to cause renal damage.

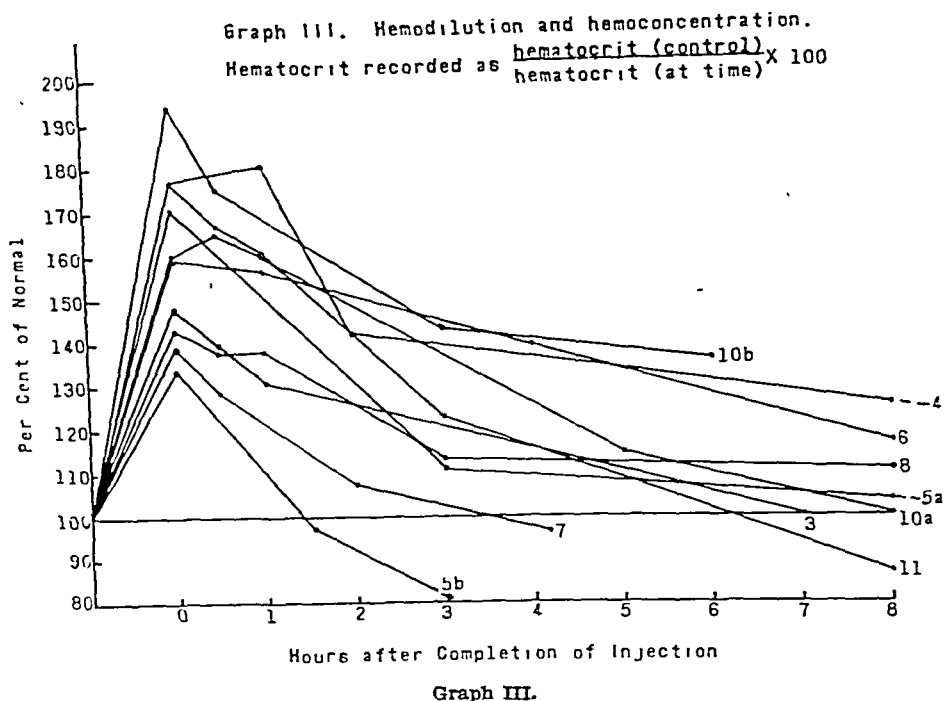
Graph II. Initial plasma hemoglobin concentration related to dose of hemoglobin.



Graph II.

There are many factors affecting the disappearance of hemoglobin from the circulation. Graph III illustrates the constant occurrence of hemodilution in the experiments. The ratio of control hematocrit to hematocrit at the time is used, for it approximates the true extent of hemodilution. It may be noted that Dog 5 developed very definite hemoconcentration at three hours and died twenty minutes later. Dog 11 also developed a minor degree of hemoconcentration at eight hours, but the rest of the dogs either returned to normal or maintained some hemodilution at eight hours. When the blood for the control hematocrit

clotted, the experiment is not represented in the graph. The statement has been made that shock occurs in acute hemolytic conditions because of the sudden disappearance of a large volume of red blood cells,<sup>37</sup> but it must be remembered that the hemoglobin remains in solution for a time. Because of the osmotic effect of hemoglobin the volume of blood probably is maintained at its original level initially and only gradually decreases. Furthermore, the osmotic effect of hemoglobin probably limits the level of hemoglobin which can be attained by injection of solutions.



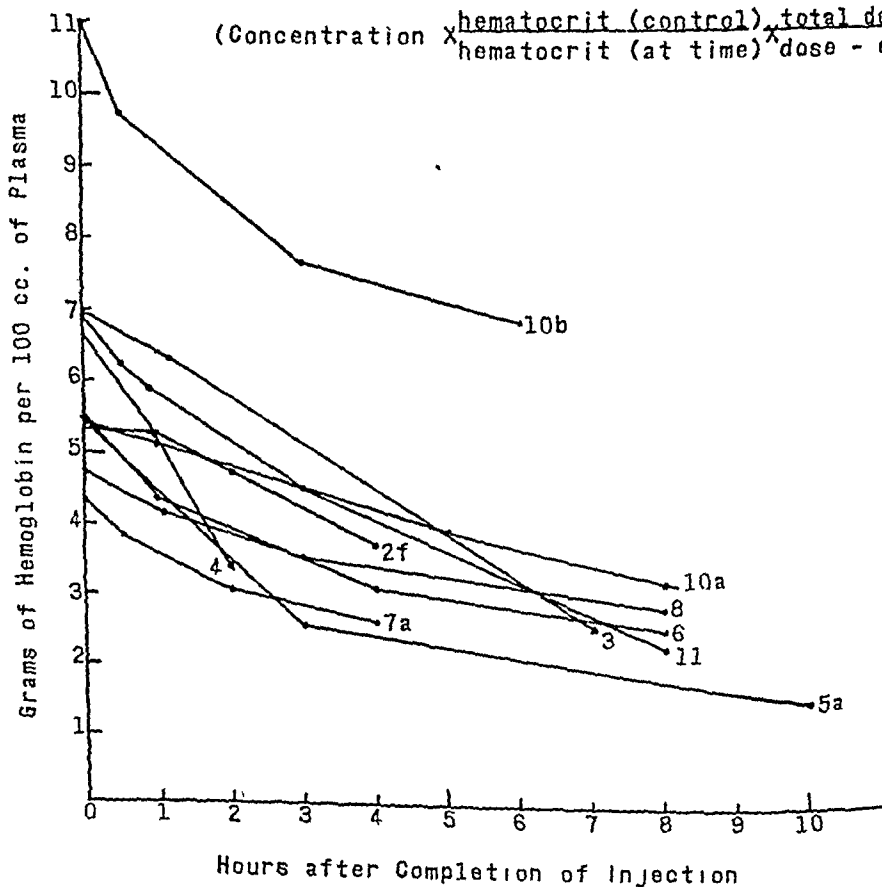
Splenic enlargement, which was evident on palpating the abdomen of all dogs immediately after completion of the injections, is also a manifestation of hemodilution. Another factor which cannot be evaluated accurately in the previously mentioned experiments is the occurrence of ascites with hemoglobin in the ascitic fluid. Ascites was present in Dogs 2 and 5 which died during the first eight hours of the experiments. In Experiment 5b it was possible to determine the hemoglobin level in the fluid a short time after the last plasma hemoglobin determination. The ascitic fluid level was only slightly lower than the plasma level. It is entirely possible that ascites was present during the early part of each experiment.

In order to eliminate or to compensate for the two major factors influencing the level of hemoglobin, namely, hemodilution and excretion of hemoglobin by the kidney, the plasma hemoglobin levels were multiplied by the ratio of control hematocrit to the hematocrit at the time and by the ratio of the initial dose of hemoglobin to the difference between the total dose and the amount excreted up

to that time. Graph IV depicts the approximately corrected hemoglobin levels. It is seen that there is a more uniform disappearance rate illustrated by this graph than by plotting the hemoglobin levels directly as in Graph I.

The body of the dog is removing hemoglobin from the circulation at a relatively constant rate. The correction for hemodilution is justified, for it gives the plasma hemoglobin level which would theoretically occur if all the hemoglobin were contained in the original blood volume. The correction for renal excretion is also justified if one is considering the ability of the organism to remove hemoglobin from the circulation, exclusive of the kidneys. The results in Experiments 4 and 10b cannot be explained readily..

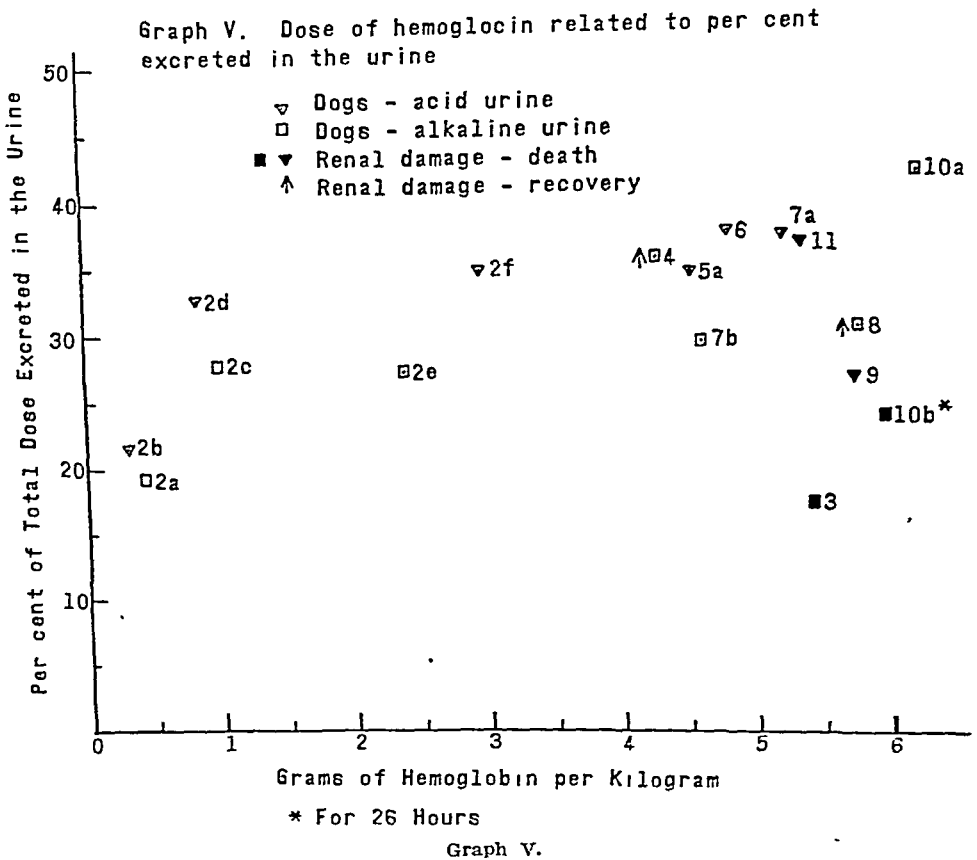
Graph IV. Plasma hemoglobin concentrations corrected for hemodilution and renal excretion.  
 (Concentration  $\times \frac{\text{hematocrit (control)}}{\text{hematocrit (at time)}} \times \frac{\text{total dose}}{\text{dose} - \text{excr.}}$ )



Graph IV.

The amount of hemoglobin excreted is dependent on several factors. Graph V shows the scatter of per cent of hemoglobin excreted in relation to dose administered. It can be seen that there is an even distribution of dogs with acid urine and those with alkaline urine when large doses were administered. It may be seen also that the lowest and the highest percentage excretion were found in

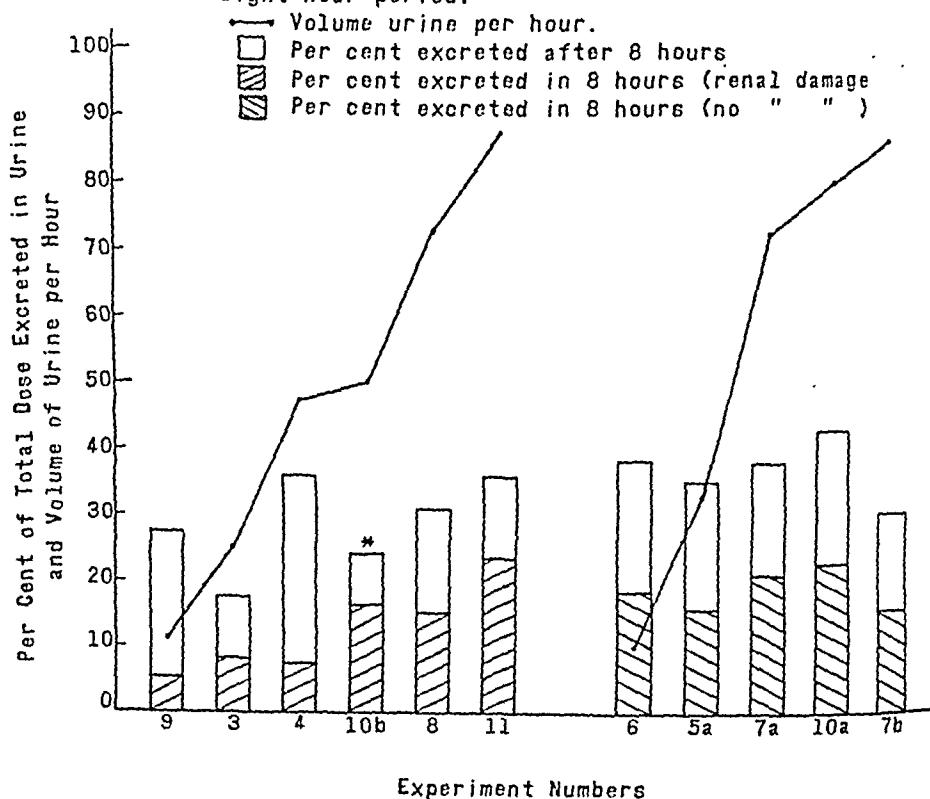
dogs with alkaline urine when the larger doses were used. As might be expected, the dogs developing renal insufficiency excreted somewhat lower percentages than the other dogs. These findings do not agree with those of De Navasquez<sup>25</sup> who used much smaller doses in rabbits, but it is believed that these results are more significant since the doses used were sufficient to cause definite renal injury in about half of the dogs. The percentage of total dose excreted is in agreement with observations of other investigators.<sup>43, 72, 73</sup>



Graph VI depicts the relationship of hemoglobin excretion in the first eight hours to total excretion and to urine volume. The amount of excretion in the first eight hours is roughly related to the development of renal insufficiency, but there is enough overlapping of values that no generalization can be made. Notice the closely comparable curves of volume of urine in the two groups.

The plasma hemoglobin level of dogs given large doses of hemoglobin does not appear to be correlated very closely with the maximum urine concentration. It is noteworthy that in Experiment 2a the maximum urine concentration was ten times that of the plasma. The maximum concentrations in the urine occurred some time after completion of the injection and usually in the period from six to eight hours afterward. This may be related to available water for urine dilution.

Graph VI. Excretion of hemoglobin in per cent of initial dose and volume of urine in first eight hour period.



\* For 26 Hours

Graph VI.

#### DISCUSSION

The results obtained by explanting one kidney of a dog and using a Silverman biopsy needle have been satisfactory. It has been possible to obtain specimens for histologic examination of the kidney at as frequent intervals as considered useful during the course of the experiments previously described. That representative samples of the kidney are obtained has been demonstrated by comparing the biopsy with autopsy specimens of the same animal a short time later. Up to now the progress of a lesion of the kidney produced by hemoglobin or other chemical agents has been studied by sacrificing animals at intervals after introducing the noxious agent or by examining the animals as they died of renal insufficiency. The method of obtaining serial biopsies should be of considerable use in the further study of induced hemoglobinuria, renal damage by chemical poisons, and experimental renal hypertension.

There are some disadvantages to the method of needle biopsy. In two animals (Dogs 6 and 11) the explanted kidney had abscesses at one pole. Since none of the rest of the kidney or of the other kidney had evidence of suppura-

tion, it is reasonable to ascribe the infection to an introduction of bacteria by the needle. More care in scrubbing and preparing the skin probably would have eliminated one of the abscesses. In the other case the novocain solution was contaminated and probably caused the infection. Small areas of infarction and later scarring of the parenchyma occurred after some of the biopsies. Hematuria resulted in some instances, and it was usually possible to identify microscopically some epithelium of the pelvis in the biopsy when hematuria had occurred. There were no serious sequelae from the hematuria. The procedure was carried out occasionally without success in obtaining an adequate specimen. It can be done by a single individual, but help to compress the kidney afterward, to hold the dog, and to steady the needle makes the procedure easier and more certain of success.

It is suggested that a record of sites of biopsy be kept on a diagram of the kidney so that the same area is not used more than once. This was not done in any of the previously mentioned experiments, but some of the unsatisfactory biopsies could have been averted and less scarring of the kidney probably would have resulted.

In all dogs which developed renal insufficiency a number of casts persisted after cessation of hemoglobinuria. The most severe renal insufficiency occurred in the dogs with most marked accumulation of casts in the tubules (Experiments 9, 10b, and 11). Dog 3 developed severe renal insufficiency but had begun to recover some renal function when it died from pneumonia. The number of casts was almost as great as in the previously mentioned three instances. Dog 8 developed renal insufficiency lasting a shorter time than that of Dog 3. Dog 4 developed the mildest renal insufficiency and evidence of renal damage of the group. Again, the degree of insufficiency was correlated with the accumulation of casts in the cortex and medulla of the kidneys. The amount of tubular epithelial injury was directly correlated with the number of casts. In other words, hemoglobin casts and tubular epithelial injury are factors of equal significance in the production of renal insufficiency.

The specific gravity of urine in patients with blackwater fever is said to be variable and of no significance, but an examination of the protocols of patients dying from uremia<sup>6, 95</sup> reveals a fixed specific gravity of the urine even in the face of a severe oliguria. In burned patients with uremia following hemoglobinuria<sup>61</sup> normal or large volumes of urine with fixed low specific gravity were excreted. Ross<sup>79</sup> reported a group of patients with blackwater fever who never developed anuria and yet had renal insufficiency. Fairley and Bromfield<sup>31</sup> report a patient with blackwater fever who had polyuria from the start, developed uremia and acidosis, but eventually recovered.

Patients who have developed renal insufficiency as the result of any acute hemolytic episode may recover completely without any signs of renal damage on testing years later.<sup>15, 45, 68</sup> The recovery of animals after developing severe renal insufficiency has also occurred. In the present study it has been possible to follow the course of degeneration and subsequent healing or partial healing in Dogs 3, 4, and 8. Figs. 2 to 5 reveal the gradual process of healing from a



stage of severe injury (Fig. 2) to healing of some of the tubules and atrophy of others (Figs. 4 and 5). Less marked renal damage developed in Dog 4 and a longer period of observation was possible so that it had practically normal appearing kidneys 112 days after injection of hemoglobin. In some sections of the kidney in this dog an occasional atrophic tubule and rarely a cast could be seen but, for the most part, the kidney was normal. The appearance of the sections in Dog 8 is intermediate between those of Dogs 3 and 4. In Dog 11 which lived ten days after injection, there was evidence of beginning repair of the tubules of the cortex.

The histologic changes in the various experiments correspond with those reported by other investigators<sup>6, 9, 50, 55</sup> and to the photomicrographs published by DeGowin and co-workers.<sup>24</sup> It is noteworthy, though, that dogs which had distinctly alkaline urine developed renal insufficiency of all degrees of severity and that the histologic appearance cannot be distinguished from the dogs with acid urine which developed renal injury. This is at variance with the findings of DeGowin and co-workers. Furthermore, they found only minor degenerative changes in the tubular epithelium of most of their dogs dying from renal insufficiency. Their photomicrograph showing regeneration corresponds to findings in some of the experiments mentioned in the article. Some necrosis and desquamation of tubular epithelium was noted in the kidneys of a few of their dogs.

The histologic appearance of the kidneys of the previously described dogs developing renal insufficiency are very similar to the pathologic findings in clinical cases.<sup>7, 21, 24, 45</sup> Most of the reported cases of death from renal insufficiency occur on an average of seven to ten days after the initiating hemolytic accident, so that the findings correspond to those at the height of degenerative changes in the tubular epithelium and also at a time when the number of casts in the cortex seems to be diminishing in the dog experiments. When death occurs as early as twenty-four hours after the hemolytic episode, the tubule cells already show some degenerative changes. The appearance of hemoglobin casts in the renal tubule is a constant finding in the cases of hemoglobinuria with anuria in human beings and it is therefore pathognomonic of hemoglobinuria.

In reports of clinical cases the number of casts noted was often small. However, every section of a tubule need not be occluded by a cast, for only one small segment of a tubule need be obstructed theoretically in order to interfere with the function of the individual nephron. In a section of kidney parenchyma a single nephron is cut across in literally hundreds of places. Nevertheless, tubular cell injury is the predominant lesion microscopically in some transfusion reactions.<sup>5, 24, 45</sup> No data are available on the number of casts nor does it seem possible to obtain such data. It is true that the number of casts in the kidneys of human cases, especially of transfusion reaction, is less than found in some of the dogs of my experiments.

The identity of the manifestations, and the lesions caused by transfusion reaction on the one hand and those caused by intravascular hemolysis of the patient's own blood cells on the other, is very important. One does not have to

look for some strange effect from the transfused blood, such as foreign protein or allergic or isoagglutinin effects. The changes are attributable to the hemolysis itself and hence to the products liberated from hemolyzed red blood cells.

The type of solution does not alter the effect of hemoglobinemia. Severe renal damage was produced by a solution of hemoglobin made from hemoglobin crystals in two out of three dogs. With comparable amounts of hemoglobin (over 5.0 Gm. per kilogram) hemolyzed red blood cell solutions also produced serious renal damage in three out of four dogs. The crystalline hemoglobin solution was made according to the directions of Bing<sup>11</sup> who found no impairment of renal function as measured by creatinine and para-aminohippuric acid clearances but who gave much smaller amounts of hemoglobin. The renal damage produced by the two types of solution cannot be distinguished by histologic examination of biopsy or autopsy specimens.

The possible deleterious effect of potassium in the solution can be excluded in these dogs for two reasons. In the first place, Kerr<sup>57</sup> has shown conclusively that the potassium content of dog red blood cells is no greater for practical purposes than that of dog serum. In the second place, administration of a solution of washed crystals of hemoglobin would eliminate any soluble crystalloid.

The most important single index of the probability of development of renal damage in the dogs I studied was the initial plasma hemoglobin level (Graph II). All dogs which developed serious renal insufficiency had values above 3.7 Gm. per 100 c.c. immediately after completing the injection of hemoglobin. Only Dog 8 had an initial level less than 3.7 Gm. per 100 c.c. and it developed only moderate renal damage. An evidence of the importance of the amount of hemoglobin in causing renal damage is the fact that five out of seven dogs given more than 5.0 Gm. hemoglobin per kilogram developed renal damage. Dog 4 was given less than this amount and developed renal damage but had an initial hemoglobin level above 3.7 Gm. per 100 cubic centimeters.

Whether or not oxyhemoglobin alone is the responsible agent cannot be answered from these experiments. In Experiment 10b the methemoglobin concentration and the total hemoglobin concentration were determined in all plasma samples and in the original solution. The plasma methemoglobin levels ranged from 0.704 to 0.530 Gm. per 100 cubic centimeters. The hemoglobin solution administered contained 13 per cent methemoglobin. The methemoglobin could be demonstrated easily by spectroscopy in all plasma samples studied in this experiment. It should be noted that methemoglobin appeared in the urine immediately in this experiment, but in other dogs with alkaline urine it never appeared until after an initial period of five to ten hours of observation. In the other experiments neither the hemoglobin solution nor the initial plasma specimen had enough methemoglobin to be visible by spectroscopy. It is probable, however, that methemoglobin was present in amounts less than that required for spectroscopic identification. As little as 3 per cent of total hemoglobin pigments can be identified as methemoglobin by spectroscopy.<sup>57</sup>

Bing<sup>11</sup> concluded that methemoglobin was the toxic agent in hemoglobinemic states only when the urine is acid. The absence of renal damage in dogs without

acidosis does not account for the results in Experiment 10b nor in the other experiments on dogs with an alkaline urine. Methemoglobin has not been found to exist in the plasma of patients who have undergone a hemolytic transfusion reaction or developed blackwater fever.<sup>32, 34, 39</sup> Methemoglobin has been found in the plasma of patients with hemolytic crisis from sulfonamides,<sup>37</sup> but it is probable that the sulfonamide had a direct effect in the production of methemoglobin. It does not seem possible at present to decide what importance should be ascribed to methemoglobin as the noxious agent in hemolytic conditions. Unfortunately, quantitative determinations were not made in any but the last experiment; however, in this instance it was known that methemoglobin was present in large quantities by spectroscopic examination before injecting the solution. In previous experiments equally diligent search failed to demonstrate any methemoglobin by spectroscopy. Methemoglobin in the urine is not the cause of the renal insufficiency, *per se*. In Dogs 5, 6, and 7 (Experiment 7a) no renal insufficiency developed though methemoglobin was recognized in the urine from the time of the first few hours. On the other hand, methemoglobin did not appear in the urine of Dogs 3, 4, or 8 in the same period, in sufficient amount to be recognized spectroscopically, and yet renal damage occurred in these dogs.

There is some evidence from clinical cases to support the thesis that the plasma hemoglobin level and/or the amount of hemoglobin liberated are the important factors. Bordley<sup>13</sup> reviewed fifteen cases of hemolytic transfusion reactions. He found that the five patients who recovered received an average of 314 c.c. of blood. Ten patients who died received an average of 564 cubic centimeters. No patient receiving less than 350 c.c. died and none receiving more than 540 c.c. recovered. There have been reports of deaths occurring almost immediately after administering less than 100 c.c. of blood,<sup>92</sup> but such cases may well have a different significance than those developing renal insufficiency after transfusion reaction.

In cases of hemolytic transfusion reaction which I previously<sup>36</sup> reported the only patient who developed anuria was one who had the highest level of hemoglobin a day after the transfusions. In the protocols of patients with blackwater fever reported by Foy and Kondi,<sup>50</sup> Fairley and Bromfield,<sup>31</sup> and Yorke and associates<sup>95</sup> the patients with the highest plasma levels were the ones who developed oliguria and anuria. In the cases of hemolytic anemia with hemoglobinuria described by Fox and Ottenberg<sup>37</sup> one patient had a plasma level of 1.6 Gm. per 100 cubic centimeters. This patient died from pneumonia but had complete urinary suppression as a result of the hemoglobinuria. Another patient who died also had renal insufficiency. The plasma level of this patient was much lower than that of the patient with pneumonia and was comparable to levels in the two patients who recovered. No statement is made as to the time relationship of the individual values to onset of hemolytic anemia, so one cannot use the second case as evidence for or against the thesis.

Another important factor becomes apparent in the study of paroxysmal nocturnal hemoglobinuria. Whenever the plasma hemoglobin levels in patients

with this malady have been determined they have been relatively low. The highest recorded level which could be found was 288 mg. per 100 cubic centimeters.<sup>52</sup> Renal damage is not a feature of the disease.<sup>53</sup> It is probable that the amount of hemoglobin liberated is usually not sufficient to raise the level dangerously. Renal damage or death from anuria is not mentioned in a review of paroxysmal cold hemoglobinuria.<sup>54</sup>

The relationship of the reaction of the urine to development of renal insufficiency was the original purpose of the thesis. The original plan of conducting serial renal biopsies was to determine whether precipitation of hemoglobin occurs only when the urine is acid. The discussion of the pathologic findings in Dogs 3, 4, 8, and 10b is sufficient to refute this idea. The severest renal damage occurred in Dogs 9 and 11 with acid urine and Dog 10b with alkaline urine. Serious renal insufficiency and renal damage occurred in Dog 3 with plasma hemoglobin and total dose comparable to Dog 9; however, the dog was recovering but was still azotemic when it died of pneumonia at forty-six days. Dog 8 with an alkaline urine developed moderately severe renal damage and insufficiency when given a dose comparable to Dog 7 with an acid urine. Dog 7 did not develop renal damage. The urine in Experiment 5 was so nearly neutral that it probably should be excluded from the present discussion of relationship of urine reaction. Two animals with acid urine (Dogs 6 and 7a) and two with alkaline urine (Dogs 7b and 10a) did not develop renal damage from hemoglobinemia. From these experiments it is impossible to say categorically that alkalinizing the urine does not benefit any dog or that acidifying the urine does not harm any dog. Alkalinizing the urine certainly does not prevent the formation of hemoglobin casts in the kidney and does not prevent serious renal damage in all dogs. Under comparable conditions the dogs with acid urine and those with alkaline urine fared equally well or equally poorly, as the case may be. Severe and even fatal transfusion reactions can occur when the urine has been previously alkalinized.<sup>14, 25</sup> In crush injuries<sup>17</sup> the benefit of alkalinizing the urine has not been definitely established but may have merit.

The results of DeGowin and co-workers<sup>23, 24</sup> are not strictly comparable to these experiments. The dogs given the acid diet may have had urine with lower pH (pH values not recorded), and their dogs with alkaline urine actually had neutral or slightly acid urine at times and slightly alkaline urine at other times. Some of the dogs with alkaline urine developed uremia of considerable proportions but recovered. Another difference is that their dogs were not anesthetized.

Cohn and Edsall<sup>19</sup> have summarized the available information regarding the solubility of hemoglobin in relation to pH. The isoelectric point of hemoglobin is pH 6.6. The solubility of hemoglobin is at its minimum at this pH. Sodium and potassium chloride do not lead to a marked decrease in the solubility of hemoglobin or albumin in their saturated solutions. Sodium sulfate is one of the most effective salting out agents. Horse and dog hemoglobin are less soluble than human hemoglobin. This information, of course, does not take into consideration the possible effect of methemoglobin or hematin formation in acid

urine, but it is important to realize that hemoglobin is least soluble at about pH 6.5. Insufficient observations were made on the presence or absence of the manifestations of shock which may have occurred in some of the dogs. The influence of shock induced by continuous administration of histamine<sup>28</sup> for one-half to one hour is an interesting observation. No functional impairment of the kidney could be detected in spite of continuous anuria for the period of lowered blood pressure. Observations of the urine flow in the present study indicate that at no time during the first eight hours after injection did the blood pressure drop low enough to cause complete cessation of urine flow. Renal blood flow may have decreased enough to allow excessive concentration of urine in the tubules.

Yuile and co-workers<sup>27</sup> have recently reported results of some very important studies on the effect of preliminary ischemia on renal damage due to hemoglobin. They injected hemoglobin solutions in doses ranging from 1.25 to 2.32 Gm. into rabbits weighing from 2.15 to 3.2 kilograms. When the tubules of the kidneys had been previously damaged by a short period of complete ischemia or the administration of sodium tartrate, hemoglobin precipitated in the tubules of the damaged kidneys but not in normal kidneys. When the urine was acid hemoglobin casts were numerous and persistent and were associated with renal functional disturbances, in contrast to the lack of such disturbances when the urine was alkaline. These observations may have an important bearing on the puzzling problem of the relation of shock and anoxia to the development of the crush syndrome, "transfusion kidney," and related lesions.

#### SUMMARY AND CONCLUSIONS

A method of obtaining serial biopsies of the same kidney of a dog has been described. By explanting one or both kidneys to the subcutaneous space of the lumbar region the kidney becomes available for biopsy with a needle biopsy apparatus or for obtaining blood samples from the renal veins. Satisfactory biopsies were obtained. It is suggested that the technique may be used to advantage for studying other problems related to experimental injury of the kidney by various agents.

When 1 Gm. of hemoglobin or more per kilogram of body weight is injected into dogs, 25 to 40 per cent is excreted by the kidney. The remaining hemoglobin is destroyed in the reticulo-endothelial system or the circulating blood with the production of bilirubin and hemosiderin. When excretion and hemodilution are corrected for, the rate of disappearance of hemoglobin is found to be more uniform during the first eight hours than is apparent from the plasma hemoglobin concentrations alone. In other words, hemoglobin is destroyed at a fairly uniform rate by the dog.

The amount of hemoglobin excreted is approximately the same when the urine is alkaline as when it is acid. This is true with relatively large doses of hemoglobin and at comparable plasma hemoglobin concentrations. There are considerable variations in maximum concentrations of hemoglobin in the urine of dogs with comparable doses and plasma concentrations.

In all dogs which developed renal insufficiency, the following two pathologic findings occurred together: (1) A large number of hemoglobin casts appeared in the loops of Henle and distal tubular segments, especially in the corticomedullary portion. The casts persisted for a long time after cessation of hemoglobinuria. The majority of tubules were occluded by casts in dogs with serious or fatal renal damage. (2) Degeneration of the tubular epithelium occurred as early as four hours, reaching a maximum at five to seven days. If the dog lived longer, gradual regeneration with eventual return to normal architecture ensued. The results of the histologic observations agree with reports of other investigators, with the exception that dogs with alkaline urine had renal damage approximately as severe as those with acid urine.

The most important indices as to development of renal damage in the dogs observed were the initial plasma hemoglobin concentration and the average of the initial and twenty-four hour plasma hemoglobin concentrations. When the initial concentration was over 3.7 Gm. per 100 c.c. of plasma or the average of the two concentrations was over 2.2 Gm. per 100 c.c., renal insufficiency developed. Dogs with values below these levels all recovered without signs of permanent renal damage due to hemoglobinuria. The renal damage was just as severe when solutions of hemoglobin crystals were used as when centrifuged solutions of washed hemolyzed red blood cells were used in similar amounts and providing comparable plasma hemoglobin concentrations. The evidence obtained in this study did not offer support for Bing's belief that the renal injury is due to methemoglobin, neither did it wholly exclude this possibility.

None of the dogs developed anuria. Renal damage was manifested by an elevation of blood urea nitrogen, albuminuria, and excretion of urine of low fixed specific gravity. Casts were found in the urine during the period of hemoglobinuria in all dogs regardless of the urine pH. Casts were also found in the urine of dogs with renal damage after cessation of hemoglobinuria.

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#### REFERENCES

1. Allen, F. M.: Experimental Studies Upon Renal Vascular Disorders, *J. Metabolic Research* 7: 217-220, 1925.
2. Amberson, W. R., Flexner, J., Steggerda, F. R., Mulder, A. G., Tendler, M. J., Pankratz, D. S., and Laug, E. P.: On the Use of Ringer-Locke Solutions Containing Hemoglobin as a Substitute for Normal Blood in Mammals, *J. Cell. & Comp. Physiol.* 5: 359-382, 1934.
3. Altschule, M. D., and Gilligan, D. R.: Acute, Massive Hemoglobinuria of Obscure Cause, With Jaundice and Anemia; Report of a Case With Clinical and Hematologic Studies and Measurements of the Blood Pigment Metabolism, *Arch. Int. Med.* 68: 957-978, 1941.
4. Aubertin, E., Lacoste, A., and Castagnou, R.: Sur le pouvoir toxique de l'hémoglobine administrée par voie veineuse chez le chien, *Compt. rend. Soc. de biol.* 132: 129-131, 1939.
- Aubertin, E., Lacoste, A., and Castagnou, R.: De la néphrite hémoglobinurique expérimentale, *Compt. rend. Soc. de biol.* 132: 132-134, 1939.

- Aubertin, E., Lacoste, A., and Castagnou, R.: *Etude comparative des gaz du sang chez des chiens, en état d'anémie aigue par saignée, traités ou non ultérieurement par une injection intraveineuse d'une solution d'homo- ou d'hétérohémoglobine, de sang de chien ou de sérum physiologique*, *Compt. rend. Soc. de biol.* 132: 141-144, 1939.
5. Ayer, G. D., and Gauld, A. G.: *Uremia Following Blood Transfusions; the Nature and Significance of Renal Changes*, *Arch. Path.* 33: 513-532, 1942.
  6. Baker, S. L., and Dodds, E. C.: *Obstruction of the Renal Tubules During Excretion of Haemoglobin*, *Brit. J. Exper. Path.* 6: 247-260, 1925.
  7. Baker, S. L.: *Urinary Suppression Following Blood Transfusion, With a Report of a Case Probably Due to Overheating Blood*, *Lancet* 1: 1390-1394, 1937.
  8. Barratt, J. O. W., and Yorke, W.: *An Investigation Into Mechanism of Production of Blackwater*, *Ann. Trop. Med.* 3: 1-257, 1909.
  9. Barratt, J. O. W., and Yorke, W.: *The Production of General Symptoms in Haemoglobinemia*, *Brit. M. J.* 1: 235-240, 1914.
  10. Bieter, R. N.: *Albuminuria in Glomerular and Agglomerular Fish*, *J. Pharmacol. & Exper. Therap.* 43: 407-412, 1931.
  11. Bing, R. J.: *The Effect of Hemoglobin and Related Pigments on Renal Functions of Normal and Acidotic Dogs*, *Bull. Johns Hopkins Hosp.* 74: 161-175, 1944.
  12. Borchardt, W., and Tropp, C.: *Experimentelle Beiträge zur Symptomatologie der akuten Bluterfallskrankheiten: Schwarzwasserfieber, paroxysmale Hämoglobinurie usw.*, *Klin. Wchnschr.* 7: 1136-1137, 1928.
  13. Bordley, J.: *Reactions Following Transfusion of Blood With Urinary Suppression and Uremia*, *Arch. Int. Med.* 47: 288-315, 1931.
  14. Brainerd, H. H.: *Renal Insufficiency Following Transfusion of Compatible Blood*, *Am. J. Obst. & Gynec.* 40: 142-144, 1940.
  15. Butler, B. C., Danforth, D. N., and Scudder, J.: *The Rh Factor in Intragroup Blood Transfusion Reactions*, *Surg., Gynec. & Obst.* 78: 610-617, 1944.
  16. Bywaters, E. G. L., and Dibble, J. H.: *The Renal Lesion in Traumatic Anuria*, *J. Path. & Bact.* 54: 111-120, 1942.
  17. Bywaters, E. G. L.: *Ischemic Muscle Necrosis; Crushing Injury, Traumatic Edema, the Crush Syndrome, Traumatic Anuria, Compression Syndrome; a Type of Injury Seen in Air Raid Casualties Following Burial Beneath Débris*, *J. A. M. A.* 124: 1103-1109, 1944.
  18. Cabot Case No. 25222, *New England J. Med.* 220: 928-931, 1939.
  19. Cohn, E. J., and Edsall, J. T.: *Proteins, Amino Acids, and Peptides as Ions and Dipolar Ions*, New York, 1943, Reinhold Publishing Corporation, pp. 598, 604-606.
  20. Culbertson, C. G., and Ratcliffe, A. W.: *Reaction Following Intra-group Blood Transfusion; Irregular Agglutinin Demonstrated by Sensitive Centrifuge Test Method*, *Am. J. M. Sc.* 192: 471-475, 1936.
  21. Daniels, W. B., Leonard, B. W., and Holtzman, S.: *Renal Insufficiency Following Transfusion; Report of 13 Cases*, *J. A. M. A.* 116: 1208-1215, 1941.
  22. DeGowin, E. L., and Baldrige, C. W.: *Fatal Anuria Following Blood Transfusions. Inadequacy of Present Tests for Compatibility*, *Am. J. M. Sc.* 188: 555-560, 1934.
  23. DeGowin, E. L., Osterhagen, H. F., and Andersch, M.: *Renal Insufficiency From Blood Transfusion. I. Relation to Urinary Acidity*, *Arch. Int. Med.* 59: 432-444, 1937.
  24. DeGowin, E. L., Warner, E. D., and Randall, W. L.: *Renal Insufficiency From Blood Transfusion. II. Anatomic Changes in Man Compared With Those in Dogs With Experimental Hemoglobinuria*, *Arch. Int. Med.* 61: 609-630, 1938.
  25. De Navasquez, S.: *The Excretion of Haemoglobin, With Special Reference to the "Transfusion" Kidney*, *J. Path. & Bact.* 51: 413-425, 1940.
  26. Drabkin, D. L., Wideman, A. H., and Landow, H.: *The Fate of Hemoglobin Injected Into the Blood Stream*, *Am. J. M. Sc.* 190: 856, 1935.
  27. Duesberg, R.: *Ueber die biologischen Beziehungen des Hämoglobins zu Bilirubin und Hämatin bei normalen und pathologischen Zuständen des Menschen*, *Arch. f. exper. Path. u. Pharmacol.* 174: 305-327, 1934.
  28. Eggleton, M. G., Richardson, K. C., Schild, H. O., and Winton, F. R.: *Renal Damage Due to Crush Injury and Ischemia of the Limbs of the Anesthetized Dog*, *Quart. J. Exper. Physiol.* 32: 89-106, 1943.
  29. Entz, F. H., and Huggins, C. B.: *Production of an Extracutaneous Kidney*, *Proc. Soc. Exper. Biol. & Med.* 28: 559-560, 1931.
  30. Evelyn, K. A., and Malloy, H. T.: *Microdetermination of Oxyhemoglobin, Methemoglobin, and Sulfhemoglobin in a Single Sample of Blood*, *J. Biol. Chem.* 126: 655-662, 1938.
  31. Fairley, N. H., and Bromfield, R. J.: *Laboratory Studies in Malaria and Blackwater Fever. II. Blackwater Fever; Haemoglobinaemia*, *Tr. Roy. Soc. Trop. Med. & Hyg.* 28: 141-156, 1934.
  - Fairley, N. H., and Bromfield, R. J.: *Laboratory Studies in Malaria and Blackwater Fever. III. A New Blood Pigment in Blackwater Fever and Other Biochemical Observations*, *Tr. Roy. Soc. Trop. Med. & Hyg.* 28: 307-334, 1934.

32. Fairley, N. H.: Methaemalbumin in Man (Pseudo-methaemoglobin), *Proc. Roy. Soc. Med.* 32: 1278-1280, 1939.
33. Fairley, N. H.: The Fate of Extracorporeal Circulating Haemoglobin, *Brit. M. J.* 2: 213-217, 1940.
34. Fairley, N. H.: Methaemalbumin. I. Clinical Aspects, *Quart. J. Med.* 10: 95-114, 1941.  
Fairley, N. H.: Methaemalbumin. II. Its Synthesis, Chemical Behaviour and Experimental Production in Man and Monkeys, *Quart. J. Med.* 10: 115-138, 1941.
35. Flink, E. B., and Watson, C. J.: A Method for the Quantitative Determination of Hemoglobin and Related Heme Pigments in Feces, Urine, and Blood Plasma, *J. Biol. Chem.* 146: 171-178, 1942.
36. Flink, E. B.: Blood Transfusion Studies. I. Differentiation of Hemolytic From Non-hemolytic Transfusion Reactions, *J. LAB. & CLIN. MED.* 31: 1067-1073, 1946.
37. Fox, C. L., and Ottenberg, R.: Acute Hemolytic Anemia From the Sulfonamides, *J. Clin. Investigation* 20: 593-602, 1941.
38. Foy, H., and Kondi, A.: Blackwater Fever in Macedonia, *Tr. Roy. Soc. Trop. Med. & Hyg.* 31: 123-138, 1937.
39. Foy, H., and Kondi, A.: Spectrographic Analysis of Pigments in Serum and Urine of Blackwater Fever, *Tr. Roy. Soc. Trop. Med. & Hyg.* 32: 49-65, 1938.
40. Foy, H., Altman, A., Barnes, H. D., and Kondi, A.: Anuria, With Special Reference to Renal Failure in Blackwater Fever, Incompatible Transfusions, and Crush Injuries, *Tr. Roy. Soc. Trop. Med. & Hyg.* 36: 197-238, 1943.
41. Foy, H., Gluckman, J., and Kondi, A.: Pigment Metabolism and Renal Failure in Acute Sulfonamide Hemolysis Resembling Blackwater Fever, *Tr. Roy. Soc. Trop. Med. & Hyg.* 37: 303-319, 1944.
42. Gabriele, D. J.: The Effect of Kidney Position on the Renal Blood Flow and Function, *Am. J. M. Sc.* 204: 227-232, 1942.
43. Gilligan, D. R., Altschule, M. D., and Katersky, E. M.: Studies of Hemoglobinemia and Hemoglobinuria Produced in Man by Intravenous Injection of Hemoglobin Solutions, *J. Clin. Investigation* 20: 177-187, 1941.
44. Gilligan, D. R., and Blumgart, H. L.: March Hemoglobinuria; Studies of the Clinical Characteristics, Blood Metabolism and Mechanism, With Observations on 3 New Cases, and Review of Literature, *Medicine* 20: 341-395, 1941.
45. Goldring, W., and Graef, I.: Nephrosis With Uremia Following Transfusion With Incompatible Blood, *Arch. Int. Med.* 58: 825-845, 1936.
46. Ham, T. H.: Studies in Destruction of Erythrocytes. I. Chronic Hemolytic Anemia With Paroxysmal Nocturnal Hemoglobinuria: An Investigation of the Mechanism of Hemolysis, With Observations of 5 Cases, *Arch. Int. Med.* 64: 1271-1305, 1939.
47. Hardin, R. C.: Reactions From Universal Donor Blood, *J. LAB. & CLIN. MED.* 31: 472, 1946.
48. Hesse, E., and Filatov, A.: Experimentelle Untersuchungen über das Wesen des hämolytischen Shocks bei der Bluttransfusion und die therapeutische Beeinflussung desselben. I. Die Nierenfunktionsstörungen im akuten Experiment, *Ztschr. f. d. ges. exper. Med.* 86: 211-230, 1933.
49. Hesse, E.: Ueber die Verwendung des sogenannten Universalspenders bei der Bluttransfusion, *Deutsche Ztschr. f. Chir.* 245: 371-382, 1935.
50. Hesse, E.: Ueber das Wesen und die Behandlung des hämolytischen Shocks nach Bluttransfusion im Lichte experimenteller und klinischer Forschung, *Beitr. z. klin. Chir.* 163: 390-405, 1936.
51. Hoffbauer, F. W.: Personal communication.
52. Hoffman, B. J., and Kracke, R. R.: Chronic Hemolytic Anemia With Paroxysmal Nocturnal Hemoglobinuria, *J. LAB. & CLIN. MED.* 28: 817-828, 1941.
53. Iljin, W.: Experimentelle Untersuchungen über das Wesen des hämolytischen Shocks bei der Bluttransfusion. III. Experimentelle Beobachtungen der Nierentätigkeit nach Einführung von heterogenem und autohämolyisiertem Blut, *Arch. f. klin. Chir.* 181: 240-249, 1934.
54. Iljin, W., and Mincey, A.: Ueber das Wesen des hämolytischen Shocks bei der Bluttransfusion. VII. Ueber die Wirkung kleiner Mengen heterogenen Blutes auf die Nierenfunktion beim Versuchstier, *Arch. f. klin. Chir.* 184: 542-548, 1936.
55. Irsilger, F. J.: Uramie nach Bluttransfusion, *Zentralbl. f. Chir.* 58: 1682-1689, 1931.
56. Johnson, R. A., and Conway, J. F.: Urinary Suppression and Uremia Following Transfusion of Blood, *Am. J. Obst. & Gynec.* 26: 255-260, 1933.
57. Kerr, S.: Studies on the Inorganic Composition of Blood. IV. The Relationship of Potassium to Acid-Soluble Phosphorus Fractions, *J. Biol. Chem.* 117: 227-235, 1937.
58. Lacoste, A., Aubertin, E., and Castagnou, R.: Observations sur l'élimination rénale de l'hémoglobine, *Compt. rend. Soc. de biol.* 132: 135-136, 1939.  
Lacoste, A., Aubertin, E., and Castagnou, R.: Sur la destinée des produits d'élimination hémoglobiniques immobilisés dans les reins et sur les réactions histopathologiques liées à cette immobilisation, *Compt. rend. Soc. de biol.* 132: 137-138, 1939.



59. Levy, J.: *Über die Nierenveränderungen bei experimenteller Hämoglobinurie*, Arch. f. klin. Med. 81: 359-382, 1904.
60. Lindau, A.: Reaktionen nach Bluttransfusion; eine ätiologische und pathologisch anatomische Studie, Acta path. et microbiol. Scandinav. 5: 382-427, 1928.
61. Lison, L.: Zur Frage der Ausscheidung und Speicherung des Hämoglobins in der Amphibienniere, Beitr. z. path. Anat. u. z. allg. Path. 101: 94-108, 1938.
62. Loesch, J.: Further Observations in Experimental Nephritis, Arch. Path. 4: 495-496, 1927.
63. Loesch, J.: Ein Beitrag zur experimentellen Nephritis und zum arteriellen Hochdruck, Zentralbl. f. inn. Med. 54: 145, 177, 1933.
64. Mackenzie, G. M.: Paroxysmal Hemoglobinuria; a Review, Medicine 8: 159-191, 1929.
65. Macgraith, B. G., and Harvard, R. L.: Dangers of Intensive Alkali Treatment in Blackwater Fever, Lancet 2: 338-339, 1944.
66. Mandelbaum, H.: Hemolytic Reaction Following Blood Transfusion; Report of a Case of Intra-group Incompatibility, Ann. Int. Med. 12: 1699-1708, 1939.
67. Mason, J. B., and Mann, F. C.: The Effect of Hemoglobin on the Volume of the Kidney, Am. J. Physiol. 98: 181-185, 1931.
68. McCandless, H. G.: A Hemolytic Blood Transfusion Reaction With Oliguria, J. A. M. A. 105: 952-954, 1935.
69. Monke, J. V., and Yuile, C. L.: The Renal Clearance of Hemoglobin in the Dog, J. Exper. Med. 72: 149-165, 1910.
70. Newman, W. V., and Whipple, G. H.: Hemoglobin Injections and Conservation of Pigment by Kidney, Liver and Spleen; the Influence of Diet and Bleeding, J. Exper. Med. 55: 637-652, 1932.
71. Olson, W. H., and Neeches, H.: Experimental Anuria in Burns, J. LAB. & CLIN. MED. 30: 371, 1915.
72. O'Shaughnessy, L., Mansell, H. L., and Slome, D.: Haemoglobin Solution as a Blood Substitute, Lancet 2: 1068-1069, 1939.
73. Ottenberg, R., and Fox, C.: The Rate of Removal of Hemoglobin From the Circulation and Its Renal Threshold in Human Beings, Am. J. Physiol. 123: 516-525, 1938.
74. Payne, R. V.: Acute Hemolytic Anaemia; Death After Transfusion, Guy's Hosp. Rep. 84: 65-71, 1934.
75. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry. Vol. II. Methods, Baltimore, 1932, Williams & Wilkins Company, pp. 558-559.
76. Ponfick, E.: Experimentelle Beiträge zur Lehre von der Transfusion, Virchow's Arch. f. path. Anat. 62: 273-335, 1875.
77. Reid, W. L.: Changes in the Volume of the Kidney in the Intact Animal; a Plethysmographic Study, With Especial Reference to Diuretics, Am. J. Physiol. 90: 157-167, 1929.
78. Rhoads, C. P.: A Method for Explantation of the Kidney, Am. J. Physiol. 109: 324-328, 1934.
79. Ross, G. R.: Researches on Blackwater Fever in Southern Rhodesia. No. 6 of the Memoir Series of the London School of Hygiene and Tropical Medicine. Part II, Chap. V. The Urine in Blackwater Fever, London, 1932, London School of Hyg. and Trop. Med., pp. 164-196.
80. Sellards, A. W., and Minot, G. R.: Injection of Hemoglobin in Man and Its Relation to Blood Destruction, With Special Reference to the Anemias, J. Med. Research 34: 469-494, 1916.
81. Shen, S. C., Ham, T. H., and Fleming, E. A.: Studies on the Destruction of Red Blood Cells. III. Mechanism and Complications of Hemoglobinuria With Thermal Burns, New England J. Med. 229: 701-713, 1943.
82. Stewart, S. G.: Acute Renal Insufficiency Following Blood Transfusion, M. Clin. North America 15: 553-559, 1931.
83. Terplan, K. L., and Javert, C. T.: Fatal Hemoglobinuria With Uremia From Quinine in Early Pregnancy, J. A. M. A. 106: 529-532, 1936.
84. Tripoli, C. J., and Fader, D. E.: The Differential Diagnosis of Certain Diseases of the Liver by Means of Punch Biopsy, Am. J. Clin. Path. 11: 516-527, 1941.
85. Wakeman, A. M., Morell, C. A., Eisenman, A. J., Sprunt, D. L., and Peters, J. P.: The Metabolism and Treatment of Blackwater Fever, Am. J. Trop. Med. 12: 407-439, 1932.
86. Webster, M. D., Engel, F. L., Laug, E. P., and Amberson, W. R.: The Influence of pH Upon the Elimination of Hemoglobin by Perfused Frog's Kidney, J. Cell. & Comp. Physiol. 5: 399-413, 1934.
87. Wendel, W. B.: Methemoglobin Determination, J. LAB. & CLIN. MED. 24: 96-101, 1938.
88. Werner, H.: Schwarzwasserfiebernieren mit besonderer Berücksichtigung der Therapie der Anurie, Arch. f. Schiff- u. Tropen-Hyg. 11: 687-688, 1907.

89. Wesselkin, P., Lindenbaum, J., and Kartashevskiy, N. G.: Experimentelle Untersuchungen über das Wesen des hämolytischen Shocks bei der Bluttransfusion. II. Die Rolle des Zentralnervensystems in der Pathogenese der Gefäßstörungen beim hämolytischen Shock, *Arch. f. klin. Chir.* 181: 227-239, 1934.
90. Whipple, G. H.: The Pathology of Blackwater Fever, *Am. J. Trop. Med.* 7: 1-14, 1927.
91. Wildegans, H.: Die Todesfalle nach Bluttransfusionen, *Deutsche med. Wchnschr.* 56: 2031-2034, 1930.
92. Witts, L. J.: A Note on Blood Transfusion, With an Account of a Fatal Reaction, *Lancet* 1: 1297-1299, 1929.
93. Witts, L. J.: The Paroxysmal Hemoglobinurias, *Lancet* 2: 115-120, 1936.
94. Yorke, W., and Nauss, R. W.: The Mechanism of the Production of Suppression of Urine in Blackwater Fever, *Ann. Trop. Med.* 5: 287-312, 1911.
95. Yorke, W., Murgatroyd, F., and Owen, D. U.: Observations on Five Cases of Blackwater Fever, *Tr. Roy. Soc. Trop. Med. & Hyg.* 23: 335-372, 1930.
96. Yuile, C. L.: Hemoglobinuria, *Physiol. Rev.* 22: 19-31, 1942.
97. Yuile, C. L., Gold, M. A., Hinds, E. G.: Hemoglobin Precipitation in Renal Tubules, *J. Exper. Med.* 82: 361-374, 1945.
98. Yuile, C. L., and Clark, W. F.: Myohemoglobinuria; A Study of the Renal Clearance of Myohemoglobin in Dogs, *J. Exper. Med.* 74: 187-196, 1941.
99. Yuile, C. L., Steinman, J. F., Hahn, P. F., and Clark, W. F.: The Tubular Factor in Renal Hemoglobin Excretion, *J. Exper. Med.* 74: 197-202, 1941.

# THE TREATMENT OF PERNICIOUS AND RELATED ANEMIAS WITH SYNTHETIC FOLIC ACID

## I. OBSERVATIONS ON THE MAINTENANCE OF A NORMAL HEMATOLOGIC STATUS AND ON THE OCCURRENCE OF COMBINED SYSTEM DISEASE AT THE END OF ONE YEAR

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THE synthetic *Lactobacillus casei* factor (pteroylglutamic acid), commonly known as folic acid, is acknowledged a potent agent in causing hematologic remissions in pernicious anemia and related macrocytic anemias.<sup>1-5</sup> These include the macrocytic anemias of sprue, pregnancy, and nutritional macrocytic anemia. Many clinical and biochemical problems arose following the initial hematologic experiments. The present study was undertaken in November, 1945, to answer two of the clinical questions: (1) Will folic acid control the anemia of pernicious anemia and sprue for long periods of time as successfully as liver extract? (2) Will folic acid prevent the development of the neurologic manifestations of pernicious anemia or control them after they have appeared? A long period of observation is required to obtain the answers. This article constitutes a progress report at the end of the first year of investigation.

### MATERIAL AND METHODS

The twenty-eight subjects comprising the group studied were patients from the medical wards and dispensary, Cincinnati General Hospital, and patients referred to us by private physicians, usually because of sensitivity to liver extract. Twenty-one patients had pernicious anemia which had been controlled satisfactorily from two to seventeen years by injection of liver extract; three had pernicious anemia in relapse; two had sprue which had been poorly controlled by liver extract; one had nutritional macrocytic anemia; and one had macrocytic anemia and diarrhea secondary to an ileosigmoidostomy performed in 1937. Pernicious anemia was diagnosed by the following findings: macrocytic hyperchromic anemia, leucopenia, polymorphonuclear leucocyte hypersegmentation, thrombocytopenia, megaloblastic arrest of the erythrocyte series in the bone marrow, and absence of free hydrochloric acid in the gastric secretion after histamine stimulation on at least two occasions. The diagnosis of sprue was made by the presence of steatorrhea, flat glucose tolerance curve, hypocalcemia, macrocytic anemia, megaloblastic arrest of the erythrocytes in the bone marrow, free hydrochloric acid in the gastric juice after histamine, and absence of evidence for pancreatic insufficiency. The patient with nutritional macrocytic anemia had free HCl in the gastric secretions and a megaloblastic type of bone marrow and for many years had eaten a diet deficient in protein and vitamins of the B complex. Fifteen of the twenty-four subjects with pernicious anemia

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had had disease of the posterior columns, lateral columns, or peripheral nerves at some time in the past, but in only one instance was there any evidence of active neurologic disease when folic acid therapy was begun. Folic acid was given orally to each patient, usually in doses of 30 mg. three times a week but in several instances in doses as low as 10 mg. or as high as 50 mg. daily. Patients were visited in their homes to insure that the medication was taken as directed. All liver therapy was discontinued but no attempt was made to modify the usual diets of the patients in their homes. Those who were brought into the hospital in relapse or for special study were given a diet from which meat, liver, and glandular products were excluded.

Complete medical and neurologic histories were obtained and physical and neurologic examinations performed on each subject at the beginning of the study. The examinations were repeated every three months, or more often if the condition of the patient demanded it. Erythrocyte and white blood cell counts were done on peripheral blood with equipment certified by the United States Bureau of Standards. Hemoglobin was determined as acid hematin using the Klett photoelectric colorimeter; hematocrit determinations were made on oxalated venous blood (4 mg. potassium oxalate and 6 mg. ammonium oxalate per 5 c.c.) by the method of Wintrobe,<sup>9</sup> and reticulocyte and platelet counts were made by the wet technique using Dameshek's method.<sup>10</sup> All hematologic tests were performed daily while the patients were hospitalized and biweekly or monthly while they were followed in the clinic. Sternal bone marrow aspirations were performed on all patients who were observed in hematologic relapse. Cover slip preparations for cytologic study of peripheral blood and bone marrow were stained with Wright-Giemsa stain.

Analysis of the gastric secretions after histamine stimulation was performed in each patient included in this study.

#### HEMATOLOGIC OBSERVATIONS

Seventeen of the twenty-one subjects with pernicious anemia in remission who received folic acid for approximately one year were maintained in a hematologic state equivalent to that achieved previously by adequate amounts of refined liver extract (Table I). In Cases 1 and 2 there was a fall in the erythrocytes and hemoglobin after nine to twelve months and in Cases 3 and 7 there was an increase. Minor fluctuations in the hematologic values occurred in all patients. Inspection of the statistical data for all twenty-one patients revealed no significant change for the group in erythrocytes and hemoglobin over the values at the beginning of folic acid therapy. Most of the patients noted an increase in appetite and weight. Three subjects with pernicious anemia in relapse (Cases 21, 23, and 24) had satisfactory initial hematologic responses following administration of 10 mg. folic acid daily. Two of the patients (Case 23, Fig. 1 and protocol and Case 21, Fig. 2 and protocol) were not maintained in remission on this dose, and as neurologic symptoms developed they required increasingly larger doses of folic acid to maintain the hematologic remission. The third patient with pernicious anemia in relapse (Case 24, Fig. 3 and protocol) treated with 10 mg. folic acid daily had a satisfactory hematologic remission following a peak reticulocytosis of 26 per cent on the eighth day and has maintained this remission for twelve months on 50 mg. daily.

The subject with nutritional macrocytic anemia (Case 27) received 10 mg. folic acid orally per day for four months. Reticulocytes rose to 11.6 per cent on the fifth day of treatment and erythrocytes and hemoglobin increased gradually from 2.6 millions per cubic millimeter and 14.3 Gm. per 100 c.c. to 4.7 millions and 15.7 Gm. in four months. Although folic acid therapy was discontinued

TABLE I. SUMMARY OF MAINTENANCE STUDIES WITH FOLIC ACID IN TWENTY-EIGHT PERSONS WITH MACROCYTIC ANEMIA; COMPARISON OF HEMATOLOGIC AND NEUROLOGIC STATUS OF EACH PATIENT AT BEGINNING OF EXPERIMENTAL PERIOD AND AT LATEST EXAMINATION

CASE	P.A.	AGE	SEX	DIAGNOSIS	YEAR ESTAB- LISHED	U.S.P. LIVER EXTRACT PER WEEK	FOLIC ACID DOSAGE (MG./ WEEK)	FOLIC ACID NO.	INITIAL ERYTH- ROCYTE COUNT (MIL- LIONS)	LATEST ERYTH- ROCYTE COUNT (MIL- LIONS)	INITIAL HEMO- GLOBIN (GM./ 100 C.C.)	LATEST HEMO- GLOBIN (GM./ 100 C.C.)	INITIAL HEMAT- OCRIT (PER CENT)	LATEST HEMAT- OCRIT (PER CENT)	COMBINED SYSTEM DISEASE		
															STATUS BEFORE LIVER EX- TRACT	STATUS BEFORE FOLIC ACID	STATUS WITH FOLIC ACID
1	J.S.	56	M	Pernicious anemia	1934	10	90	13	4.30	3.25	14.5	12.3	43	40	Mild	None	No change
2	F.R.	75	M	Pernicious anemia	1935	10	90	13	4.50	3.32	13.9	12.8	41	38	Mild	None	No change
3	I.H.	70	F	Pernicious anemia	1929	10	90	13	3.91	3.86	11.4	13.0	39	40	Mild	None	No change
4	C.S.	56	M	Pernicious anemia	1944	60	90	12	4.82	4.81	15.5	14.7	49	48	Severe	Mild	No change
5	M.H.	55	F	Pernicious anemia	1943	10	90	13	4.47	4.54	13.0	14.8	45	43	Mild	None	No change
6	A.P.	46	M	Pernicious anemia	1944	10	90	11	4.62	4.96	16.3	17.7	51	48	Moder- ate	None	No change
7	E.W.	35	M	Pernicious anemia	1941	15	90	13	3.38	5.05	11.0	15.1	46	47	Mild	None	No change
8	R.P.	70	F	Pernicious anemia	1939	10	90	12	4.29	3.80	14.5	13.8	41	42	Mild	Mild	No change
9	M.S.	44	F	Pernicious anemia	1944	?	90	12	4.74	4.79	15.7	14.9	46	42	None	None	No change
10	D.K.	82	F	Pernicious anemia	1935	10	90	13	4.42	4.43	12.5	13.0	43.5	44	Mild	Mild	No change
11	R.H.	63	M	Pernicious anemia	1942	30	90	13	3.90	4.23	12.0	13.0	45	44	Mild	Mild	No change
12	N.C.	72	F	Pernicious anemia	1943	5	90	13	4.60	4.59	14.0	14.6	45	48	Mild	None	No change
13	N.T.	71	F	Pernicious anemia	1938	10	90	13	4.65	4.66	14.0	13.0	45	41	Severe	Moder- ate	No change
14	L.E.	67	F	Pernicious anemia	1935	30	90	13	4.74	4.57	14.5	13.7	40	42	Moder- ate	Mild	No change
15	L.W.	75	M	Pernicious anemia	1932	5	90	13	4.48	4.35	14.5	14.6	45	40	None	None	No change

16	A. K.	55	F	Pernicious anemia	1942	10	90	13	4.39	4.15	14.0	13.7	40	43	None	None	No change
17	R. S.	65	F	Pernicious anemia	1936	None for 1 yr.	90	6	4.11	4.72	14.8	15.3	43	47	None	None	No change
18	L. H.	63	F	Pernicious anemia	1938	10	90	13	5.20	5.18	15.0	14.6	50	46	None	None	No change
19	C. D.	62	M	Pernicious anemia	1938	3	90	13	4.50	4.83	14.0	15.0	46		Moderate	Stationary	No change
20	J. F.	65	M	Pernicious anemia	1940	7	90	10+	3.65	3.50	12.9	13.9	37.5	39	None	None	Slowly progressive
21	M. V.	59	F	Pernicious anemia	1936	None for 9 mo.	70	8+	1.07	2.35	4.0	10.0			None	None	Severe
22	L. F.	62	F	Pernicious anemia	1935	7.5	90	9+	4.21	4.20	15.7	15.8	44	42	None	None	Slowly progressive
23	N. G.	63	F	Pernicious anemia	1946	0	35	6	1.57	3.57	7.2	12.8	19		None	None	Severe
24	F. C.	66	F	Pernicious anemia	1945	0	70	13	1.22	3.79	4.7	14.5	15.5	42	Mild? Nurtis	Stationary	No change
25	B. A.	64	M	Sprue	1941	15	90	13	5.00	5.16	15.3	17.4	51	51			
26	A. F.	35	M	Sprue	1944	10	630	12	4.54	4.43	15.6	16.4	47	48			
27	J. I.	74	M	Nutritional macrocytic anemia	1946	0	70	7	2.60	4.94	14.3	15.3	38	49			
28	D. J.	41	M	Secondary macrocytic anemia (folic acid deficiency)	1937	10	525	7	4.37	4.28	16.5	14.6	47	46			

Time of last blood counts and neurologic examinations.

## NG 63 ? PERNICIOUS ANEMIA, 1 YEAR

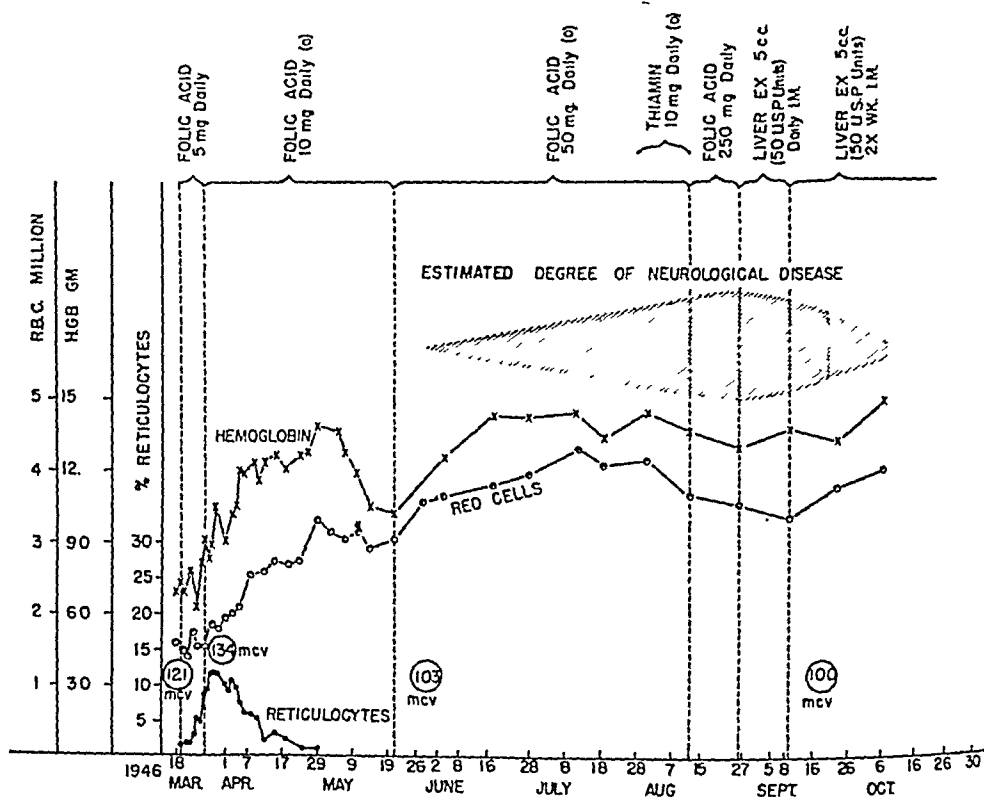


Fig. 1.

*Case 23.*—N. G., a 63-year-old white woman, had anorexia and mild dyspepsia for a year and had lost 25 pounds in weight. There were no neurologic symptoms. The dietary excluded milk and eggs and the patient seldom ate meat.

Physical examination revealed only pallor of the mucous membranes, a waxy color of the skin, and a few red papillae at the tip of the tongue. There were no neurologic abnormalities.

Laboratory studies on March 19, 1946, showed: erythrocytes, 1.57 millions; hemoglobin, 7.2 Gm.; leucocytes, 4,950; reticulocytes, 1.6 per cent; platelets, 314,000; mean corpuscular volume, 121; mean corpuscular hemoglobin, 46; mean corpuscular hemoglobin concentration, 38; a normal leucocyte differential count; bone marrow, megaloblastic arrest of the erythrocyte series; normal gastrointestinal series; histamine refractory achlorhydria.

Folic acid orally, 5 mg. per day for eleven days, was begun on March 19, 1946. On the twelfth day, March 31, 1946, the dose of folic acid was increased to 10 mg. daily and maintained for approximately two months. In five weeks (April 25, 1946) the erythrocytes were 3.31 millions, the hemoglobin 13.8 grams. The patient felt entirely well, and although redness of the papillae of the tip of the tongue waxed and waned there was no soreness. The appetite was excellent and there was a weight gain of 22 pounds. After two months (May 19, 1946) hematologic values had fallen slightly and a neurologic examination showed: hyperactive knee and ankle jerks, positive Romberg sign, and slight reduction of vibration perception at the ankles. The only subjective complaint was of attacks of weakness in the right leg. The dose of folic acid was increased at this time to 50 mg. orally daily. There was a gradual

(Continued on opposite page.)

after four months, the patient's diet was improved to include foods rich in "extrinsic factor(s)." Three months after discontinuing folic acid there has been no relapse and none is to be expected.

The two subjects with sprue (Cases 25 and 26) were maintained in normal hematologic equilibrium. Remarkable improvement in diarrhea which had not been controlled by liver extract was noted in Case 26. Bowel movements decreased from five to six daily to one daily and weight increased from 127 to 147 pounds in two months. When folic acid therapy was discontinued for three months, mild glossitis appeared but cleared rapidly when folic acid therapy was reinstituted. The second subject with sprue (Case 25) continued in a satisfactory state of remission and the weight increased from 119 to 126 pounds.

The patient with macrocytic anemia secondary to ileosigmoidostomy (Case 28) was maintained in as normal a hematologic state by folic acid therapy as by crude and refined liver extract therapy. This person had had six to eight bowel movements daily since 1937 in spite of liver extract and was weak and distressed by abdominal distention. After three months of folic acid therapy the bowel movements had decreased to three daily, the weight had increased from 131 to 136 pounds, abdominal distention was partially relieved, and the patient was able again to play eighteen holes of golf without fatigue. (The improvement of a diarrhea essentially of a mechanical variety was not expected and no explanation is evident. Maintenance of a normal blood picture, however, suggests efficient absorption of folic acid from the small intestine.)

#### NEUROLOGIC OBSERVATIONS

Only one patient had subjective complaints or objective evidence of active central or peripheral nervous system degeneration prior to the administration of folic acid. This patient (Case 24, Fig. 3 and protocol) had numbness and

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rise in erythrocytes and hemoglobin during the next months (July 13, 1946) to 4.16 millions and 14.2 Gm., respectively. Within a week of the discovery of positive neurologic signs the patient noticed numbness and tingling in the hands and feet and unsteady gait. The neurologic signs and symptoms progressed rapidly in spite of a final increase in the dose of folic acid to 250 mg. orally daily for two weeks (Aug. 13, 1946). The patient was afraid to get out of bed for fear of falling. Numbness and tingling in the hands and feet were severe. Examination showed ataxia in arms and legs, hypalgesia over hands and feet, vibration perception 50 per cent reduced at the ankles and knees and 25 per cent in the fingers, and perception of position slightly reduced in the toes, deep reflexes hyperactive and the snout reflex positive; there were no plantar reversal signs or clonus.

At this point five months after continuous therapy with folic acid in increasing dosage and after a slight fall in erythrocytes and hemoglobin to 3.57 millions and 12.8 Gm., respectively, folic acid was discontinued (Aug. 27, 1946). Refined liver extract, 50 U.S.P. units, was administered intramuscularly daily for two weeks and twice weekly thereafter. In two weeks the patient felt much better. The paresthesias had almost disappeared. She walked well but with steppage gait. The Romberg test was normal, and there was no ataxia. Pain, light touch, temperature, and position perception were normal. Vibration perception improved but after six weeks of liver therapy was still reduced approximately 30 per cent at the ankles, 20 per cent at the knees, and 10 per cent in the fingers. The deep reflexes remained hyperactive. All redness disappeared from the tongue. The erythrocytes and hemoglobin rose very slowly in six weeks to 4.13 millions and 14.1 Gm., respectively.



## M.V. 59 ♀ PERNICIOUS ANEMIA 10 YEARS

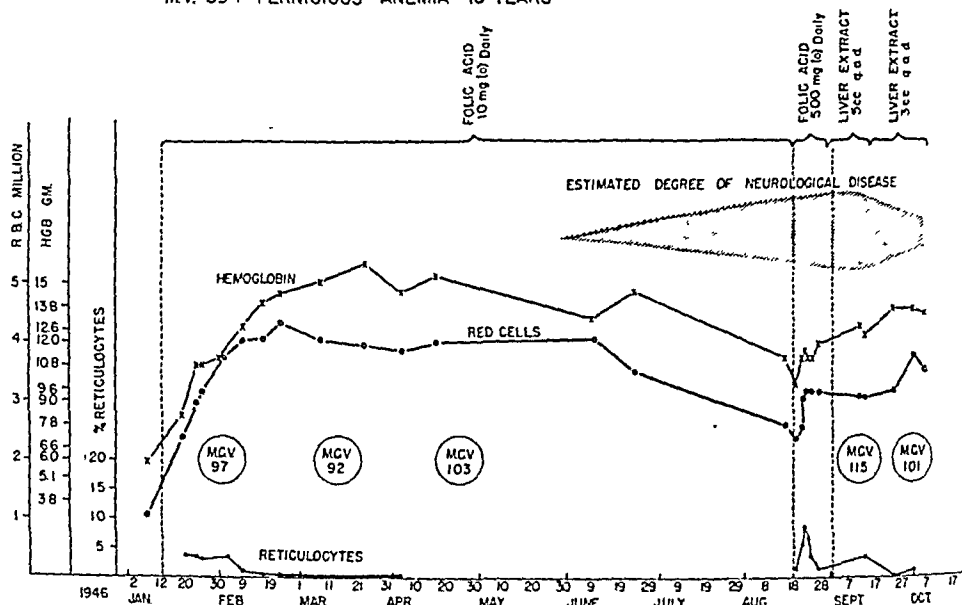


Fig. 2.

**Case 21.**—M. V., a 59-year-old white woman, had Addisonian pernicious anemia of ten years' duration. Treatment with liver extract had been sporadic because of hypersensitivity reactions. Symptoms of relapse were weakness, pallor, and anorexia. On Jan. 15, 1946, she had received a hypodermic injection which was said to be iron cacodylate.

The patient was small, thin, and pallid. Glossal papillae were atrophied. There was no evidence of brain, spinal cord, or peripheral nerve disease.

Laboratory studies revealed: histamine refractory achlorhydria; normal gastrointestinal series; erythrocytes, 1.07 millions; hemoglobin, "less than 6 Gm.," white blood cell count, 6,250.

**Course:** On Jan. 12, 1946, folic acid 10 mg. orally daily was begun. The blood picture improved and was well maintained on 10 mg. folic acid through the fifth month. At that time, however, stiffness in the knees and numbness and tingling in the fingers occurred. Examination showed: Romberg positive, ataxia in the legs, pain and light touch sensations preserved, position perception impaired in the toes, and vibration not felt at the ankles and approximately 75 per cent reduced at the knees. The patient left town without notice but had a supply of folic acid adequate for 10 mg. daily for two months. She returned at the end of this period unable to walk without assistance. She had evidently taken the folic acid but the exact amount could not be determined. Mental deterioration was obvious. The patient seldom completed a sentence and her memory was poor.

Examination showed: pallor, a few red papillae at the tongue tip; motor weakness at the ankles and knees; vibration perception absent at the knees and ankles, reduced in pelvis, normal in hands; perception of position of the toes absent; marked ataxia in the legs and ankle clonus; knee and ankle jerks hyperactive; and plantar reversal signs positive. Erythrocytes were 2.59 millions, hemoglobin 10 grams. The bone marrow showed moderate megaloblastic arrest in the erythroid series. The patient was hospitalized and given 250 mg. folic acid twice daily for twelve days. There was a moderate reticulocytosis to 8.6 per cent on the sixth day and a slight increase in erythrocytes but no change in the neurologic disease. Refined liver extract, 50 U.S.P. units daily for seven days and then every other day, induced neurologic improvement in ten days (disappearance of clonus and a normal Chaddock

(Continued on opposite page.)

tingling in the hands and feet, stocking type of hypesthesia to touch, and pin prick and diminished knee and ankle reflexes, interpreted as signs of peripheral neuritis. While receiving folic acid, 10 mg. daily, the signs of peripheral neuritis increased. Remission or progression in these signs could not be correlated with increases or decreases in the dosage of folic acid and for the last nine months the patient's complaints and neurologic abnormalities have been essentially the same as when treatment was begun.\*

After five to eight months on folic acid therapy, four subjects with pernicious anemia (Cases 20 to 23) noted the gradual onset of numbness and tingling sensations in the hands and feet, unsteadiness of gait, and moderate stiffness in the extremities. Neurologic signs appeared after the hematologic status had reached or was near normal and gradually progressed until there was definite evidence of combined system disease. The neurologic abnormalities implicated the posterior columns and peripheral nerves in four of the subjects, lateral columns in three, and cerebral cortex in one subject. None of these persons had neurologic involvement of any type when pernicious anemia was diagnosed initially or when folic acid therapy was begun. When signs of nervous system degeneration were unequivocal, usually indicated by the appearance of a positive Romberg sign, the extensor response on plantar stimulation, loss of sense of position and of perception of vibration in the feet, and hypesthesia and hypalgesia in fingers and toes, each of these four patients received 50 to 500 mg. folic acid orally per day for ten to forty days. These large doses of folic acid resulted in no subjective or objective improvement. Refined liver extract (10 U.S.P. units per cubic centimeter), 3 to 5 c.c. given intramuscularly per day, was begun after it was obvious that amelioration of the neurologic signs and symptoms would not occur or when progression of the disease was so rapid as to endanger the patient's future well-being. Within ten days thereafter improvement in the neurologic status began. The dose of liver extract was reduced to 3 to 5 c.c. on alternate days, and convalescence is progressing satisfactorily in all four patients. The progression of neurologic disease in the two patients most severely affected is indicated in Figs. 1 and 2. Shown in Fig. 3, Case 24, is the course of the patient with peripheral neuritis in whom the exacerbations and remissions seemed to occur independent of folic acid treatment. Accompanying each figure is the appropriate case history.

#### DISCUSSION

As yet too little time has elapsed to make sweeping statements regarding the effectiveness of folic acid as maintenance therapy for the patient with pernicious

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response). In two months she was clear mentally and could walk without aid but with a wide base. All plantar reversal signs had disappeared. There was slight swaying with the Romberg test. The position of the toes was perceived after maximal stimulation. Vibration was perceived at the knees but not at the ankles. The erythrocyte count and hemoglobin had gradually increased to 4.26 millions and 14.4 Gm., respectively.

\*Since this article was submitted for publication this patient has developed definite posterolateral sclerosis, has been treated intensively with liver extract, and has shown a moderate remission.

## M.V. 59 ♀ PERNICIOUS ANEMIA 10 YEARS

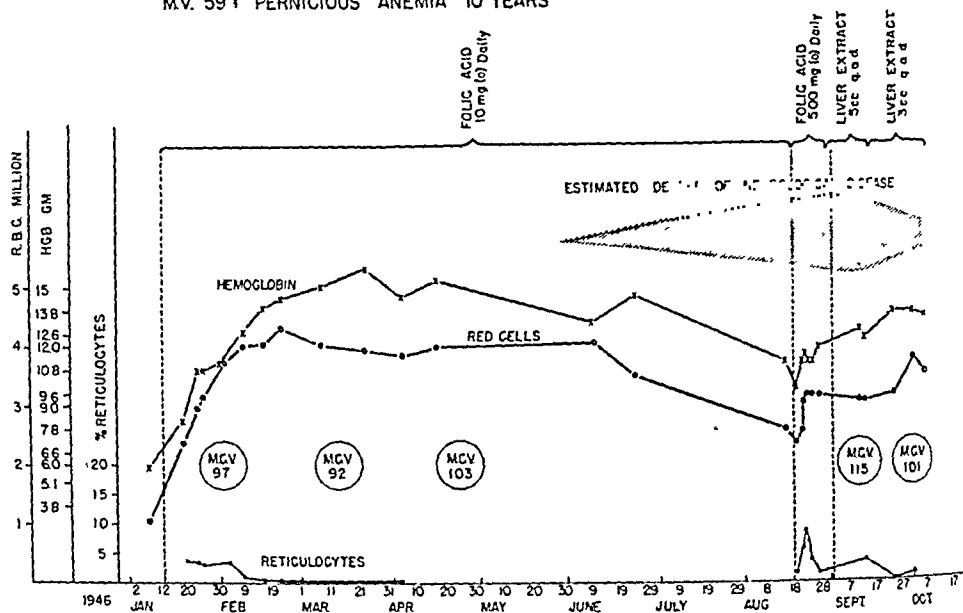


Fig. 2.

*Case 21.*—M. V., a 59-year-old white woman, had Addisonian pernicious anemia of ten years' duration. Treatment with liver extract had been sporadic because of hypersensitivity reactions. Symptoms of relapse were weakness, pallor, and anorexia. On Jan. 15, 1946, she had received a hypodermic injection which was said to be iron cacodylate.

The patient was small, thin, and pallid. Glossal papillae were atrophied. There was no evidence of brain, spinal cord, or peripheral nerve disease.

Laboratory studies revealed: histamine refractory achlorhydria; normal gastrointestinal series; erythrocytes, 1.07 millions; hemoglobin, "less than 6 Gm.," white blood cell count, 6,250.

*Course:* On Jan. 12, 1946, folic acid 10 mg. orally daily was begun. The blood picture improved and was well maintained on 10 mg. folic acid through the fifth month. At that time, however, stiffness in the knees and numbness and tingling in the fingers occurred. Examination showed: Romberg positive, ataxia in the legs, pain and light touch sensations preserved, position perception impaired in the toes, and vibration not felt at the ankles and approximately 75 per cent reduced at the knees. The patient left town without notice but had a supply of folic acid adequate for 10 mg. daily for two months. She returned at the end of this period unable to walk without assistance. She had evidently taken the folic acid but the exact amount could not be determined. Mental deterioration was obvious. The patient seldom completed a sentence and her memory was poor.

Examination showed: pallor, a few red papillae at the tongue tip; motor weakness at the ankles and knees; vibration perception absent at the knees and ankles, reduced in pelvis, normal in hands; perception of position of the toes absent; marked ataxia in the legs and ankle clonus; knee and ankle jerks hyperactive; and plantar reversal signs positive. Erythrocytes were 2.59 millions, hemoglobin 10 grams. The bone marrow showed moderate megaloblastic arrest in the erythroid series. The patient was hospitalized and given 250 mg. folic acid twice daily for twelve days. There was a moderate reticulocytosis to 8.6 per cent on the sixth day and a slight increase in erythrocytes but no change in the neurologic disease. Refined liver extract, 50 U.S.P. units daily for seven days and then every other day, induced neurologic improvement in ten days (disappearance of clonus and a normal Chaddock

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anemia. It is impossible to predict how long a patient who has had adequate treatment with liver extract previously may go untreated without suffering a relapse.<sup>11</sup> The length of time before relapse depends upon such variables as the amount of stored erythrocyte maturation factor, the amount of intrinsic factor (however minute) produced by the stomach, the amount of extrinsic factor(s) in the diet, and the occurrence of infections and degenerative diseases. It is a well-known fact that adequately treated patients with pernicious anemia may suffer relapses within two to six months after liver extract is discontinued or they may remain in good health for as long as fifteen years.<sup>12</sup> Observations over a period of one year fall far short of being optimal; yet, considering the size of the group they suggest that folic acid will, in most cases, maintain the hematologic remission initiated by itself or by liver extract. There is no obvious mechanism to explain the fall in erythrocyte counts and hemoglobin levels in several of the patients. There was no evidence of infection or abnormality other than pernicious anemia in these patients and no obvious change in diets or activities. The amount of folic acid which previously had induced a remission in Cases 21 and 23 later seemed to be decreasingly effective in these same patients. Speculations on the mechanisms behind this are possible but too presumptive.

It is evident that folic acid did not prevent the occurrence of combined system disease, nor did massive doses halt or reverse the degenerative process once it had begun. Liver extract on the other hand bettered the neurologic status. These observations augment the single case of treatment failure of combined system disease reported by Moore<sup>13</sup> at the 1946 Meeting of the Society for Clinical Investigation and similar observations of Bethel<sup>14</sup> and Hall<sup>15</sup> and their associates. They confirm the many rumors that synthetic folic acid has proved disappointing in combined system disease. On the other hand, Doan<sup>8</sup> has reported one subject with mild neurologic abnormalities which disappeared during therapy with folic acid. The statement is made that "Minor neurologic signs and symptoms in patients in relapse have responded as promptly and completely following *L. casei* factor supplements as with potent liver extract, and no progressive cord lesions have as yet been noted."

Similar differences of opinion were recorded from 1927 to 1935, when the effect of liver extract in combined system disease was under close scrutiny.

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illions, the hemoglobin 11.1 grams. At this time (Dec. 17, 1945) there was proximal extension of hypesthesia in the hands and appearance of stocking hypesthesia in the legs. On Jan. 31, 1946, when the erythrocyte count was 4.19 millions and hemoglobin 13.0 Gm., further extension of signs of peripheral neuritis were noted. Folic acid was again increased to 50 mg. daily and continued through Nov. 20, 1946. In five weeks the signs of neuritis had returned to the initial degree. Repeated neurologic examinations revealed no change through November, 1946. No definite signs of posterolateral sclerosis had been observed at any time and the signs of neuritis appeared and disappeared independent of folic acid therapy. In September and October, 1946, a slight fall in the hemoglobin and erythrocytes was noted and in Nov. 22, 1946, the values were erythrocytes 3.9 millions, hemoglobin 10.7 grams. This occurred during an exacerbation of bronchial asthma and continued observation is necessary for final evaluations.

## F.C. 66 ♀ PERNICIOUS ANEMIA, ASTHMA

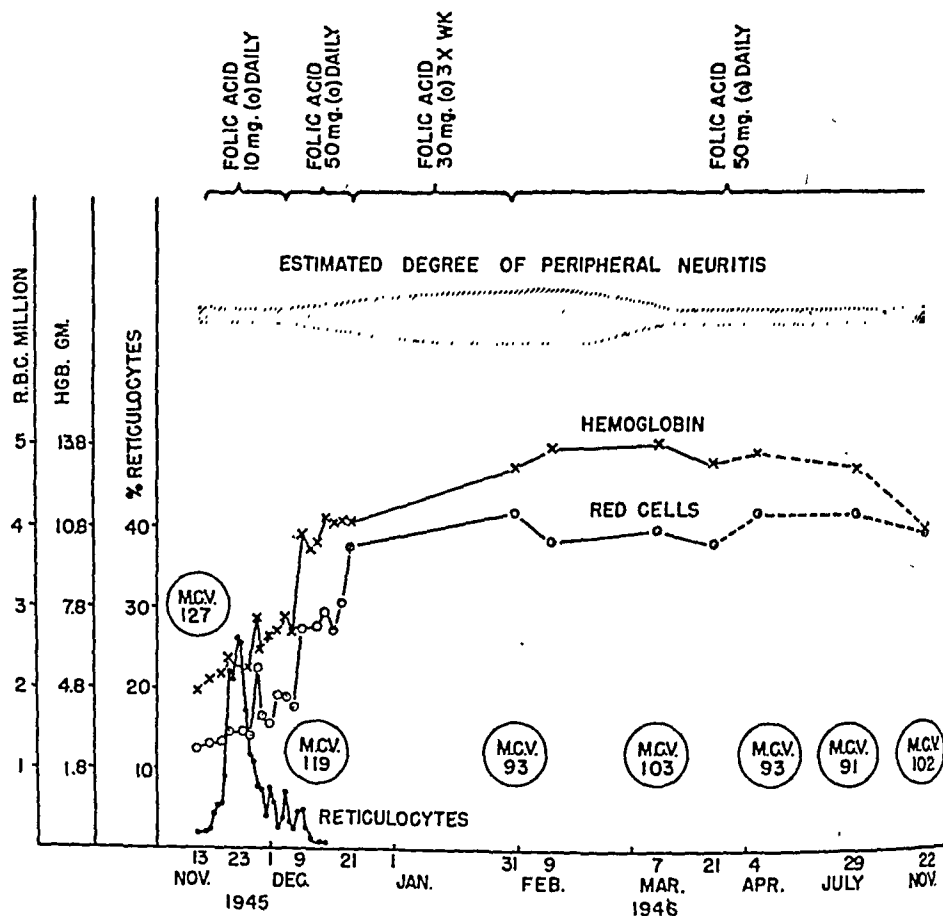


Fig. 3.

*Case 24.*—F. C., a 65-year-old white woman, complained of paresthesias in the hand and feet, intermittent glossitis, and progressive weakness for six weeks. Her two sister had pernicious anemia. The patient was pale and slightly icteric. The tongue was normal. Vibratory perception was impaired at knees and ankles; perception of position of the toe was intact; there was hyperalgesia and hypesthesia on the palmar surfaces of the hands; and knee jerks were hyperactive and ankle jerks hypoaffective.

Laboratory data revealed: erythrocyte count, 1.22 millions; hemoglobin, 4.7 Gm.; white blood count, 1,500; packed cell volume, 15 per cent; reticulocytes, 2.0 per cent; platelets 178,120; mean corpuscular volume, 127; mean corpuscular hemoglobin, 38.6; mean corpuscular hemoglobin concentration, 30.4; megaloblastic arrest in erythrocyte series in the sternum marrow; and histamine refractory achlorhydria. X-ray studies revealed a small hiatus hernia and a duodenal diverticulum.

Synthetic folic acid, 10 mg. daily, was given orally beginning on Nov. 15, 1945. Reticulocytes began to rise on the second day and reached a peak of 26 per cent on the eighth day. In twenty days the erythrocytes and hemoglobin had risen to 1.90 millions and 7.5 Gm., respectively, and the dose of folic acid was increased to 50 mg. daily for seventeen days and then reduced to 30 mg. three times a week. In another ten days the erythrocyte count was 2.4.

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7. Doan, C. A., Wilson, H. E., Jr., and Wright, C. S.: Folic Acid (L. casei Factor) an Essential Pan-Hematopoietic Factor; Experimental and Clinical Studies, Ohio State M. J. 42: 139, 1946.
8. Doan, C. A.: Folic Acid (Synthetic L. casei Factor) an Essential Pan-Hematopoietic Stimulus, Am. J. M. Sc. 212: 257, 1946.
9. Wintrobe, M. M.: Clinical Hematology, ed. 1, Philadelphia, 1942, Lea & Febiger, p. 179.
10. Dameshek, W.: Method for Simultaneous Enumeration of Blood Platelets and Reticulocytes With Consideration of Normal Blood Platelet Count in Men and Women, Arch. Int. Med. 50: 579, 1932.
11. Schwartz, S. O., and Legere, H.: Relapses in Pernicious Anemia, J. A. M. A. 124: 637, 1944.
12. Jenks, A. L., Jr.: Pernicious Anemia; Report of a Case With 15-Year Remission, J. Iowa M. Soc. 33: 112, 1943.
13. Moore, C. V., and Bierbaum, O.: Further Observations on the Antipernicious Anemia Effect of Synthetic L. casei Factor, J. Clin. Investigation (Abstr.) 25: 909, 1946.
14. Bethel, F. H., Swendseid, M. E., Bird, O. D., Brown, R. A., Meyers, M. C., and Andrews, G. A.: Further Studies on the Utilization of Pteroyl Hexaglutamyl Glutamic Acid (Vitamin B<sub>12</sub> Conjugate) in Pernicious Anemia, Proc. Central Soc. Clin. Research 19: 28, 1946.
15. Hall, B. E., Watkins, C. H., and Hargraves, M. M.: Experiences With Synthetic Folic Acid in the Treatment of Anemias of Macrocytic Type, Proc. Central Soc. Clin. Research 19: 55, 1946.
16. Minot, G. R., and Murphy, W. P.: A Diet Rich in Liver in the Treatment of Pernicious Anemia, J. A. M. A. 89: 759, 1927.

Careful studies during this period presented evidence for and against the beneficial effect of liver extract on the neurologic degeneration originally recorded by Minot and Murphy.<sup>10</sup> The effectiveness of liver extract was established only after many years of careful study and control of the potency. Therefore, final opinion must await further study of the effect of folic acid in various doses and by parenteral as well as oral routes of administration. Until a factor essential to the preservation of the central and peripheral nervous systems is found and until much more is known concerning the mode of action of folic acid in hematopoiesis, liver extract will remain the safest effective agent for the routine treatment of pernicious anemia.

#### SUMMARY

1. Folic acid in doses of 10 to 15 mg. daily or 30 mg. three times a week given orally maintained twenty-one persons with pernicious anemia in apparent hematologic remission for one year. Three patients with pernicious anemia in whom treatment was begun during a relapse responded satisfactorily to an initial daily dose of 10 mg. folic acid, but two of them required increasing amounts daily to maintain the remission; the third patient received 50 mg. daily for most of the year to determine the effect of the drug on peripheral neuritis.

2. Folic acid given orally controlled the manifestations of sprue in two persons for one year, nutritional macrocytic anemia in one person for seven months, and macrocytic anemia secondary to ileosigmoidostomy in one person as/or more effectively than liver extract.

3. Folic acid in doses of 10 to 50 mg. daily did not prevent the development of combined system disease in four persons with pernicious anemia. After combined system disease developed, folic acid in doses of 100 to 500 mg. daily given orally did not stop its progression in these persons. Liver extract, however, promoted rapid improvement.

4. These observations suggest that an unknown factor or factors in liver other than folic acid are essential for maintaining the integrity of the central and peripheral nervous systems in persons with pernicious anemia.

5. Folic acid cannot be considered satisfactory or complete treatment for pernicious anemia and should not replace potent liver extract in the routine treatment until another method for the protection of the nervous system is devised.

#### REFERENCES

1. Spies, T. D., Vilter, C. F., Koch, M. B., and Caldwell, M. H.: Observations on the Anti-anemic Properties of Synthetic Folic Acid, *South. M. J.* 38: 707, 1945.
2. Vilter, C. F., Spies, T. D., and Koch, M. B.: Further Studies on Folic Acid in the Treatment of Macrocytic Anemia, *South. M. J.* 38: 781, 1945.
3. Moore, C. V., Bierbaum, O. S., Welch, A. D., and Wright, L. D.: The Activity of Synthetic *Lactobacillus casei* Factor ("Folic Acid") as an Antipernicious Anemia Substance, *J. LAB. & CLIN. MED.* 30: 1056, 1945.
4. Spies, T. D., Lopez, G. G., Menendez, J. A., Minnich, V., and Koch, M. B.: The Effect of Folic Acid on Sprue, *South. M. J.* 39: 30, 1946.
5. Darby, W. J., and Jones, E.: The Treatment of Sprue With Synthetic *Lactobacillus casei* Factor (Folic Acid, Vitamin M), *Proc. Soc. Exper. Biol. & Med.* 60: 259, 1945.
6. Zuelzer, W. W., and Ogden, F. N.: Folic Acid Therapy in Macrocytic Anemias of Infancy, *Proc. Soc. Exper. Biol. & Med.* 61: 176, 1946.

TABLE I. RELATIVE DISTRIBUTIONS OF MANGANESE IN HUMAN BEINGS

ORGAN	PATIENT												
	6+*	4+	9	11	3+	2	7	12°	8	10	13°	5	1
liver	6.4	7.3	20.0	53.0	17.0	32.4	69.0	43.0	15.6	17.6	7.5	46.0	15.6
spleen	0.5	3.3	2.2	6.0	2.5	1.2	2.7	15.0	1.6	-	2.2	2.5	1.0
heart (ventricle)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
heart (auricle)	-	-	-	-	-	1.4	-	-	-	-	-	-	-
lung (right)	0.3	0.6	2.6	0.65	0.3	1.6	3.0	-	-	-	0.54	-	-
lung (left)	0.6	0.6	-	-	0.5	-	-	-	-	-	-	2.2	-
vertebral marrow	-	3.2	-	0.28	0.5	1.9	-	-	-	1.0	-	0.2	-
pancreas	0.8	4.9	9.4	41.0	3.5	14.5	31.0	13.0	11.3	-	7.7	29.0	-
adrenal	2.0	-	-	2.1	5.5	4.0	-	-	3.2	12.0	1.0	-	-
testis	-	-	-	0.9	-	-	-	-	1.0	-	0.9	1.8	-
kidney (right)	1.8	3.9	12.1	59.5	-	9.7	41.0	3.0	10.5	-	3.1	11.6	10.6
kidney (left)	1.7	3.1	-	-	1.5	-	-	-	-	-	-	11.6	-
muscle (striated)	0.2	-	-	-	-	-	-	-	-	-	-	1.3	-
lymph nodes:													
Pancreatic	-	-	-	-	-	4.4	-	-	2.0	-	-	-	-
Mesenteric	1.9	1.9	2.2	3.4	-	3.5	19.6	-	-	-	-	-	-
Perihepatic	-	-	-	-	-	-	-	-	2.0	-	-	-	-
Retroperitoneal	-	1.9	5.8	-	-	-	-	-	-	-	-	-	-
Periaortic	-	-	-	-	3.2	-	-	-	-	-	-	3.7	-
Hilar	-	4.4	3.1	-	4.5	-	-	-	0.5	-	-	-	-
Omental	-	-	-	-	3.9	-	-	-	-	-	-	-	-
Mediastinal	-	-	-	-	2.9	-	-	-	-	-	-	-	-
Inguinal	-	-	-	-	-	-	-	-	-	-	-	-	-
Perisplenic	-	-	5.3	-	-	-	-	-	-	-	-	5.5	-
Brain	-	-	-	-	0.1	-	-	-	-	-	-	-	-
Stomach	-	-	-	-	-	5.5	-	-	-	-	-	-	-
Jejunum	0.4	-	2.1	-	0.7	-	6.6	-	-	-	-	2.4	-
Ileum	-	-	4.2	-	-	-	-	-	-	-	-	1.5	-
Colon	-	-	1.8	-	-	-	-	-	-	-	0.23	1.2	-
Uterus	-	-	3.0	-	-	-	-	-	-	-	-	-	-
Fat:													
Omental	-	-	-	-	0.9	-	-	-	-	-	-	-	-
Perisplenic	-	-	-	-	-	-	-	-	-	-	-	-	-
Perinephric	-	-	-	-	-	-	-	-	-	-	-	1.5	-
												0.8	-

Values are in counts per minute per gram of tissue relative to heart muscle as unity. Patients are arranged in order of the increasing time between final dose and autopsy.

\*. Tracer amount only

\*. Patients received both Mn and Au; only Mn counted.

\*. Injected intraperitoneally.

given which remained in the liver, spleen, and lungs. Variations in concentration throughout a given organ make this estimate rather uncertain. This effect becomes evident upon inspection of the data in Table III, in which studies of gold distribution in four dogs are presented. The large variation in the estimated percentage of the dose retained by the liver is evident. In Dog 4 the concentration in liver and spleen is determined from multiple samples, and the data show a consistent picture of complete removal of gold by these organs alone. The variation in gold content of different samples of liver and spleen in Dog 4 is shown in Table IV. The distribution of manganese in liver, spleen, pancreas, and heart of two dogs is shown in Table V. Here, instead of determining the activity of random samples, entire organs were used.



# STUDIES OF THE DISTRIBUTION OF INTRAVENOUSLY ADMINISTERED COLLOIDAL SOLS OF MANGANESE DIOXIDE AND GOLD IN HUMAN BEINGS AND DOGS USING RADIOACTIVE ISOTOPES

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## INTRODUCTION

RECENTLY in the course of internal radiation therapy using colloidal manganese dioxide containing radioactive manganese ( $Mn^{52}$ )<sup>1</sup> an opportunity has been presented to study the distribution of small particles in human beings when introduced intravenously. Similar observations have been made using colloidal metallic gold containing the radioactive isotope  $Au^{198}$ . In several patients whose disease came to fatal termination it was possible to determine the radioactivity of various tissues taken at autopsy, permitting a relative comparison to be made of their concentrations of manganese or gold. Three patients also were studied whose life expectancy was a matter of hours. Here only tracer doses were given. One of these subjects received the material via the intraperitoneal route. The distribution studies in human beings were augmented by studies of distributions in dogs; a record was obtained also of the disappearance of colloidal gold from the circulation in one dog and in one human subject.

## RESULTS

In Table I is shown the relative distribution of radioactivity in selected tissue samples from eleven patients who had received radioactive manganese and two who received both manganese and gold. The distributions in patients receiving both isotopes were determined under conditions where the radiation from gold had disappeared. Thus only manganese is measured. Usually several doses had been given; however, due to radioactive decay, conditions were such that almost all of the radiation was due to a particular final dose. Since all values are relative some method of common comparison is required. Thus we have expressed the results in terms of the ratio of the activity per gram of tissue (wet weight) to that of the heart (muscle). Patient 6 is of particular interest since the dose was given intraperitoneally to this subject. In Table II are shown equivalent data for two patients who received gold alone. We attribute the approximate tenfold difference in numerical results primarily to a large variation in the content of the heart muscle in the two patients. In one patient we were able to determine tissue concentrations in micrograms of gold per gram of tissue. It was possible also to estimate the percentage of the dose

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TABLE III. DISTRIBUTION OF COLLOIDAL GOLD IN DOGS

ORGAN	DOG 1			DOG 2			DOG 3			DOG 4		
	A	B	C	A	B	C	A	B	C	A	B	C
Liver	2.9	2700	27%	7.3	1300	66%	11.7	25000	101%	11.5	24000	92.5%
Spleen	7.3	6800	7%	2.8	490	2.5%	1.5	3200	2.5%	10.3	21400	8.3%
Lung	0.54	50	2%	0.056	1	-	0.11	230	-	0.025	1	5.2
Heart	0.0107	1	-	0.057	1	-	0.0047	1	-	0.0048	1	0.21
Pancreas	0.010	0.93	-	0.0011	0.019	-	0.0022	0.47	-	0.001	0.21	-
Brain	0.0019	0.18	-	0.0004	0.007	-	0.0004	0.08	-	-	1.2	-
Kidney	0.072	6.7	-	0.016	0.28	-	0.013	28.0	-	0.0057	0.33	-
Gut	0.0019	1.8	-	0.0075	0.13	-	0.004	0.85	-	0.0016	-	-
Thyroid	0.0078	0.73	-	0.0056	1.0	-	0.0044	0.94	-	-	-	-
Adrenal	0.053	0.50	-	0.029	0.51	-	-	-	-	-	-	-
Marrow:												
Vertebral	0.085	7.9	-	0.044	0.77	-	0.043	9.1-	-	-	-	-
Sternal	0.020	1.9	-	0.059	1.0	-	0.096	20.0	-	-	-	-
Long bone	0.15	14.0	-	0.11	1.9	-	0.24	51.0	-	-	-	-
Lymph nodes:												
Mesenteric	0.012	1.1	-	0.0074	0.13	-	0.024	5.1	-	0.0096	2.0	-
Cervical	0.023	2.2	-	0.029	0.51	-	0.0093	2.0	-	-	-	-
Axillary	0.023	2.2	-	0.0031	0.054	-	0.012	2.6	-	0.002	0.42	-
Inguinal	-	-	-	0.0023	0.04	-	-	-	-	0.002	0.42	-
Ovaries	-	-	-	0.0047	0.082	-	-	-	-	-	-	-

A, Values are in micrograms of gold per gram of tissue.

B, Values are ratio of concentration to that of heart ventricle.

C, Values are estimated fraction of initial dose in organs of high uptake.

TABLE II. DISTRIBUTION OF COLLOIDAL GOLD IN HUMAN BEINGS

ORGAN	PATIENT 14 RELATIVE ACTIVITY	PATIENT 15 RELATIVE ACTIVITY	PATIENT 15 GOLD CONCENTRATION	PATIENT 15 ESTIMATED FRACTION OF INITIAL DOSE
Liver	29.6	800	0.76	60%
Spleen	93.0	430	0.41	3%
Heart	1.0	1.0	0.00095	-
Pancreas	-	1.4	0.0013	-
Kidney	26.0	1.9	0.0018	-
Marrow:				
Vertebral	11.0	-	-	-
Sternal	-	120.0	0.11	-
Lymph nodes:				
Axillary	2.0	-	-	-
Mesenteric	-	0.95	0.0009	-
Cervical	-	-	-	-
Periaortic	2.9	-	-	-
Lungs	3.1	46.0	0.044	2%
Adrenal	5.1	2.0	0.019	-
Testis	6.2	0.7	0.00066	-

In Patient 14 results are expressed in counts per minute per gram of tissue relative to heart muscle taken as unity.

In Patient 15 concentrations in micrograms per gram of tissue are given and estimates of the total fraction of the initial dose in certain organs are calculated.

#### THE COLLOIDAL SOLS

The manganese dioxide dispersions were prepared by oxidizing manganous chloride with potassium permanganate. Gelatin was used as the supporting colloid. The method has been described previously in more detail.<sup>1</sup> The sols were not dialyzed and there is some evidence that a small fraction of undispersed crystalloidal manganese is present.<sup>2</sup> Although precise determination of particle size was not made in each case, frequent microscopic observations of these dispersions using dark-field illumination showed that they were ultramicroscopic in size and exhibited a vigorous Brownian movement. In one case a sol was prepared for electron microscopy and a picture was taken.\* The distribution in size of the particles of this sol was determined from the electron micrographs and is shown in Fig. 1. The second maximum may be due to the interaction of excess permanganate with gelatin. In all cases these sols had a deep brown color when observed by transmitted light and a greenish cast under reflected light.

In the animal experiments quantities of the order of 5 mg. of gold or less could be dispersed with good control of particle size. The sols were prepared by reducing a solution of gold chloride with ascorbic acid. Due to the instability of ionic gold and the presence of excess ascorbic acid, the gold dispersal should certainly be complete. Although gelatin was used, it is not essential. At pH 3 to 5 a characteristic dispersion was obtained containing relatively large particles. Such a sol was pale blue by transmitted light and had a heavy brick-colored bluish under reflected light. The Tyndall beam was strong and an appreciable fraction of the scattered light was unpolarized. Under dark-field illumination the particles exhibited a relatively sluggish Brownian motion. The

\*Electron microscopy was kindly done by Dr. H. C. O'Brien, Department of Physics, University of Pittsburgh.

## ADMINISTRATION

Sols were given to the patients via the cubital vein. In most instances an isotonic saline infusion was first begun. The material was added to the infusion and washed in with further saline. In a few cases the sols were given directly by syringe. In one case serial blood samples were taken immediately following injection and at short intervals for about one hour thereafter. The disappearance of gold from the circulation is shown in Figs. 2 and 3. The pertinent clinical data for the patients studied are shown in Table VI. The number of milligrams of material given and the intervals between the last dose and autopsy are included.

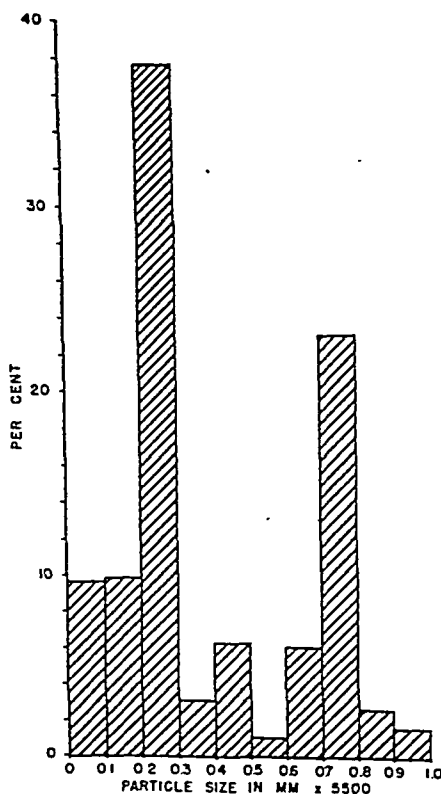


Fig. 1.—Distribution in particle size of a typical manganese dioxide sol.

The dogs were injected via the jugular vein. In all cases female mongrel animals were used. The animals were untrained and were not in a controlled state of nutrition. The animal weights, duration of life after injection, and type of sol given in each case are given in Table VII. In the case of gold each animal (Dogs 1 to 4) received the same total amount of gold, namely, 4 milligrams. A disappearance curve for one dog who received a gold sol of the large particle type is shown in Figs. 4 and 5.

TABLE IV. VARIATION IN GOLD CONCENTRATION IN LIVER AND SPLEEN OF ONE DOG

LOCATION OF SAMPLE	CONCENTRATION
Liver—tip of right central lobe	8.0
Liver—center of right central lobe	11.6
Liver—top of right central lobe	16.1
Liver—center of lobus quadratus	10.7
Liver—center of left central lobe	13.3
Liver—center of left lobe	12.6
Liver—center of left lobus Spigelii	12.6
Liver—center of right lobe	11.4
Spleen—extreme dorsal end	9.9
Spleen—one-third of total length from dorsal end	9.0
Spleen—one-third of total length from ventral end	11.2
Spleen—extreme ventral end	13.1

The notation is that of Ellenburger and Baum.<sup>2</sup>

Concentrations are in micrograms of gold per gram of tissue.

All spleen sections, 1 cm. thick, are taken transverse to the major axis of the organ.

TABLE V. DISTRIBUTION OF RADIOACTIVE MANGANESE IN DOGS

ORGAN	DOG 5			DOG 6		
	ACTIVITY	PER CENT INITIAL DOSE	RATIO TO HEART	ACTIVITY	PER CENT INITIAL DOSE	RATIO TO HEART
Liver	11.8	55.0	11.0	9.6	48.0	12.0
Spleen	1.7	1.0	1.6	4.4	2.0	5.5
Pancreas	7.1	1.3	6.6	4.4	1.5	5.5
Heart	1.07	0.4		0.8	0.2	

Activities are in counts per minute per gram.

particles were easily thrown out of solution by centrifuging under an acceleration of about 2,000 grams. A sol of this type will be referred to as a large particle sol. Under alkaline conditions (pH 8 to 10) the sols had a characteristically different appearance. They were both red by transmitted light and exhibited little or no blush. The Tyndall beam was faint but was highly polarized. Under dark-field illumination the particles showed a violent Brownian motion and could not be removed from suspension by centrifuging. This sol will be called the small particle sol. Dark-field comparison of samples of the two sols having equal concentrations of gold showed that the small particle sol contained by far the greatest average number of particles per unit volume. Electron microscope pictures of the particles in both of these sols were obtained. These pictures showed that the particles were uniform in size and roughly spheroidal in shape. The large particles were about  $200\text{ m}\mu$  in size and the small ones about one-fifth to one-tenth as large.

In therapy larger amounts of gold were dispersed at one time. Here the close control of particle size was not attained, but by adjusting the pH rough control was still possible. The therapeutic sols were dispersed under alkaline conditions and contained particles which were on the whole similar to those of the small particle sols. The color to transmitted light was initially reddish violet similar to that of blackberry juice. As time proceeded the color changed to a deeper red. Dark-field studies indicated a greater spread in particle size but a predominance of small particles. It was repeatedly verified that gold sols can be autoclaved at will. Once dispersed they maintain full stability under widely varying conditions of temperature, pH, and salt content.

## RADIOACTIVE ISOTOPES AND MEASUREMENTS OF RADIOACTIVITY

The radioactive manganese\* contained principally  $\text{Mn}^{52}$  of half-life 6.5 days. Small amounts of  $\text{Mn}^{54}$  were present. Since this isotope has a 310-day half-life, distribution data could be obtained at considerable time after the last dose of manganese. The radioactive gold† was the 2.7-day isotope  $\text{Au}^{198}$ . No long-lived activity was found after more than ten half-lives of decay.

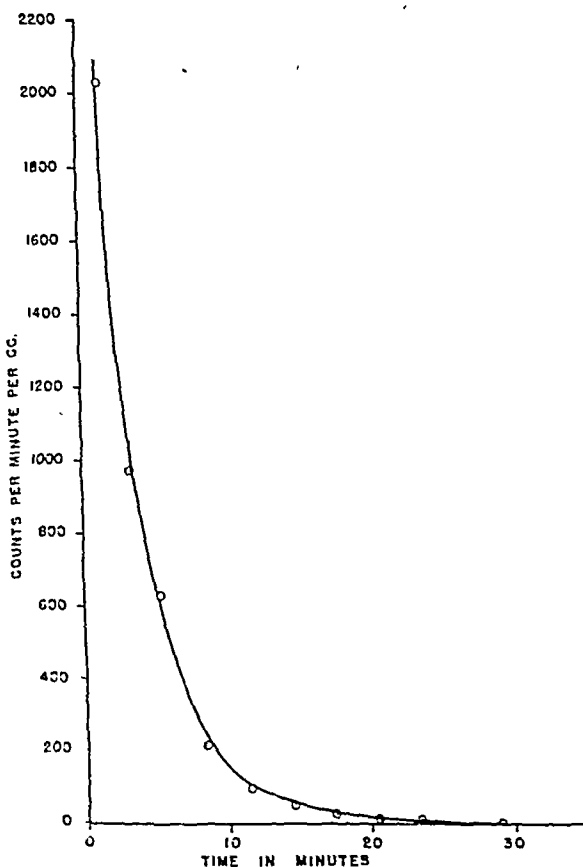


Fig. 2.

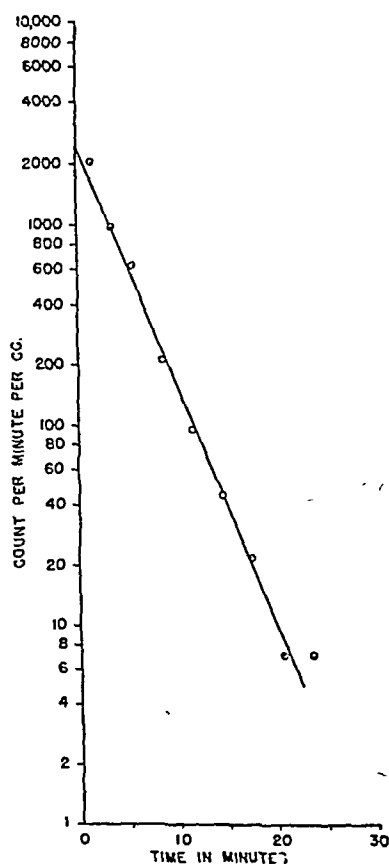


Fig. 3.

Fig. 2.—Disappearance of a therapeutic gold sol from the circulation of a human patient. (Time is measured from the end of the injection period.)

Fig. 3.—Semilogarithmic plot of the data shown in Fig. 2.

At autopsy fresh tissue samples were taken, weighed, dried overnight at  $105^{\circ}\text{C}$ . and then dry ashed in a muffle furnace at  $600$  to  $700^{\circ}\text{C}$ . A second ashing was frequently required in order to reduce the level of carbonaceous material to a negligible amount in the sample. In the case of manganese dioxide the ash was dissolved in  $\text{HCl}$ , hydrogen peroxide was added to reduce the manganese, and then each sample was transferred to a small metal

\*The radioactive manganese was furnished by the M.I.T. radioactivity center.

†The radioactive gold was obtained from the Manhattan District, Clinton Laboratories, Oak Ridge, Tenn.

TABLE VI. CLINICAL DATA, DOSAGES, AND INTERVALS BETWEEN LAST DOSE AND AUTOPSY

PATIENT	AGE	SEX	LAST DOSE IN MILLIGRAMS	TIME IN DAYS FROM LAST INJECTION OF ISOTOPE TO AUTOPSY	CLINICAL FEATURES	AUTOPSY
1	41	F	0.7* Mn 0.75 Mn	30 and 50*	Hodgkin's disease, splenomegaly; hepatomegaly, poor liver function, esophageal varices with hemorrhage	Esophageal varices with rupture, cirrhosis of liver; no Hodgkin's tissue found
2	8	M	3.0 Mn	4	Typical subacute lymphogenous leucemia	No leucemic infiltration found; bone marrow aplastic; bronchopneumonia; biliary cirrhosis
3	36	M	0.5 Mn	3½	Hypertension with rapidly progressive vascular damage; renal failure	Malignant nephrosclerosis
4	82	M	0.5 Mn	1½	Arteriosclerosis; chronic congestive heart failure (colored patient)	Cardiac hypertrophy and dilatation; pulmonary congestion; passive congestion of liver and spleen with fibrosis
5	54	M	0.3 Mn	30	Chronic lymphogenous leucemia	Chronic lymphogenous leucemia, with extensive infiltration
6	67	M	0.5 Mn	3 hr. (intraperitoneal)	Carcinoma of the rectum	Adenocarcinoma of rectum; perforation of large bowel; purulent generalized peritonitis
7	7	F	0.6 Mn	20	Acute leucemia	Aplastic bone marrow; very little evidence of leucemia infiltration
8	13	M	1.7 Mn	20	Acute leucemia	Aplastic bone marrow; no evidence of leucemia infiltration
9	10	F	3.8 Mn	1	Acute leucemia	Lymphogenous leucemia with extensive infiltration
10	45	M	0.9 Mn	20†	Carcinoma of cecum with metastases to liver	Adenocarcinoma of cecum; carcinomatosis; aplastic bone marrow
11	10	M	3.5 Mn	2	Acute leucemia	Lymphogenous leucemia with minimal infiltration; aplasia of bone marrow
12	7	M	-‡	13	Acute leucemia	Lymphogenous leucemia with extensive infiltration
13	45	M	-‡	28	Chronic myelogenous leucemia	Leucemia, probably myelogenous, with extensive infiltration
14	57	M	1.8 Au	8	Lymphoblastic lymphosarcoma	Extensive lymphosarcomatous infiltration, primary in retroperitoneal nodes and involving myocardium and abdominal viscera
15	51	M	1.5 Au	12 hr.	Hodgkin's disease	Granulocytopenia, acute; bilateral bronchopneumonia

MnO<sub>2</sub> dosages are expressed as milligrams of metallic manganese.

\*Two doses of roughly equivalent effect on tissue activity at autopsy.

†Several doses of roughly equivalent effect on tissue activity at autopsy all greater than twenty days.

‡Patients received both Mn<sup>55</sup> and Au<sup>198</sup>; only Mn<sup>55</sup> effective at autopsy.

## RADIOACTIVE ISOTOPES AND MEASUREMENTS OF RADIOACTIVITY

The radioactive manganese\* contained principally  $\text{Mn}^{52}$  of half-life 6.5 days. Small amounts of  $\text{Mn}^{54}$  were present. Since this isotope has a 310-day half-life, distribution data could be obtained at considerable time after the last dose of manganese. The radioactive gold† was the 2.7-day isotope  $\text{Au}^{198}$ . No long-lived activity was found after more than ten half-lives of decay.

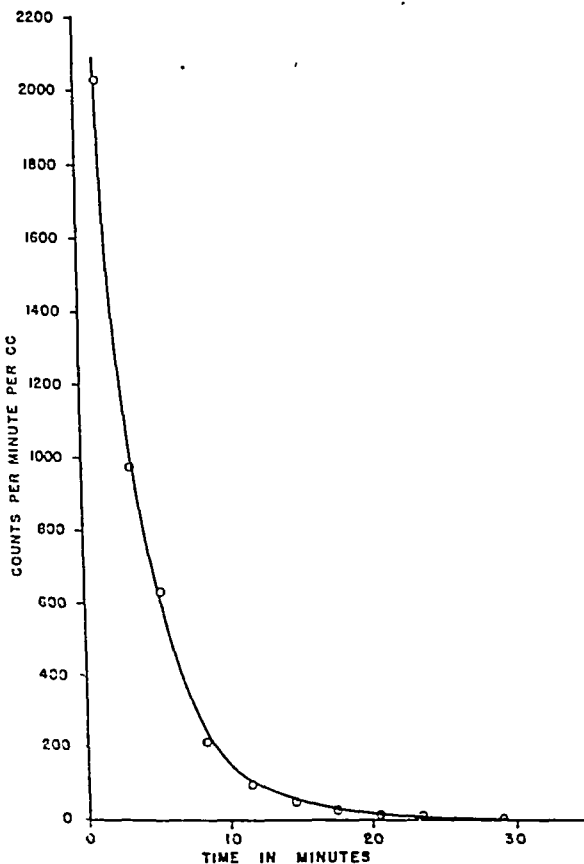


Fig. 2.

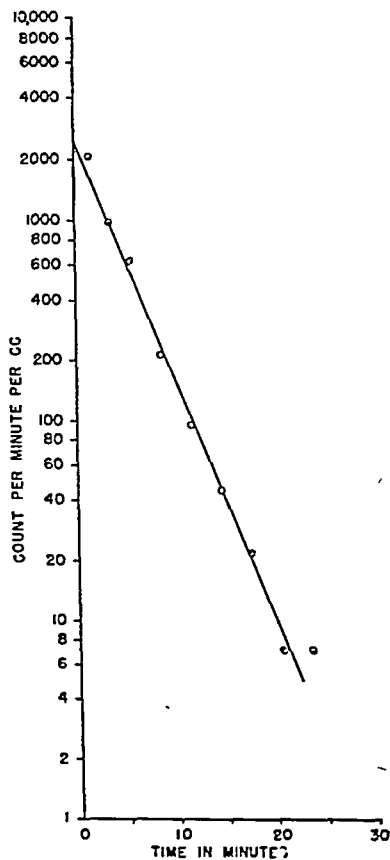


Fig. 3.

Fig. 2.—Disappearance of a therapeutic gold sol from the circulation of a human patient. (Time is measured from the end of the injection period.)

Fig. 3.—Semilogarithmic plot of the data shown in Fig. 2.

At autopsy fresh tissue samples were taken, weighed, dried overnight at  $105^{\circ}\text{C}$ . and then dry ashed in a muffle furnace at  $600$  to  $700^{\circ}\text{centigrade}$ . A second ashing was frequently required in order to reduce the level of carbonaceous material to a negligible amount in the sample. In the case of manganese dioxide the ash was dissolved in  $\text{HCl}$ , hydrogen peroxide was added to reduce the manganese, and then each sample was transferred to a small metal

\*The radioactive manganese was furnished by the M.I.T. radioactivity center.

†The radioactive gold was obtained from the Manhattan District, Clinton Laboratories, Oak Ridge, Tenn.



ointment tin<sup>4</sup> and dried. In some cases carrier manganese was added; however, no significant difference was found between samples in which this addition was made and others. In a limited number of cases check determinations of the gamma ray activity of samples were made before and after transfer from the beakers in which the ashing was conducted. Transfer was found to be complete, and negligible quantities were left behind. In the case of radioactive gold, nonradioactive gold chloride was added to the samples as a carrier in all cases. After ashing, the gold was taken into solution by adding a few drops of aqua regia. Excess acid was removed by heating before transfer was made to the cups. In the studies of disappearance of radioactivity from the circulation, the blood samples were dried directly in the cups and counted.

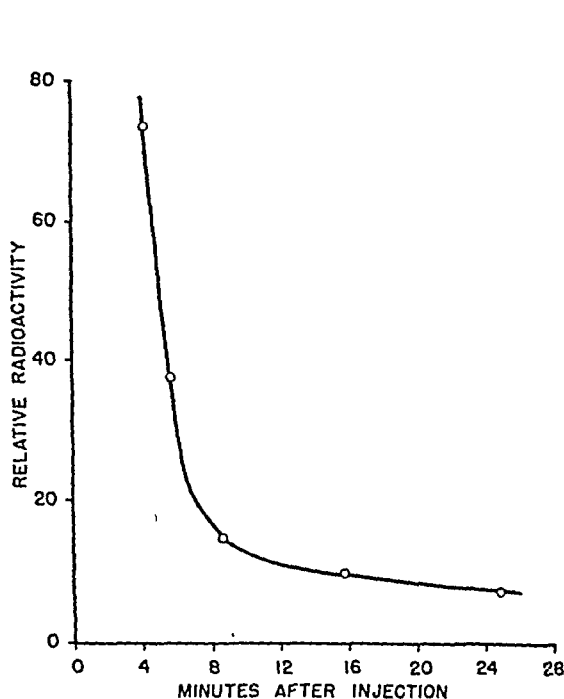


Fig. 4.

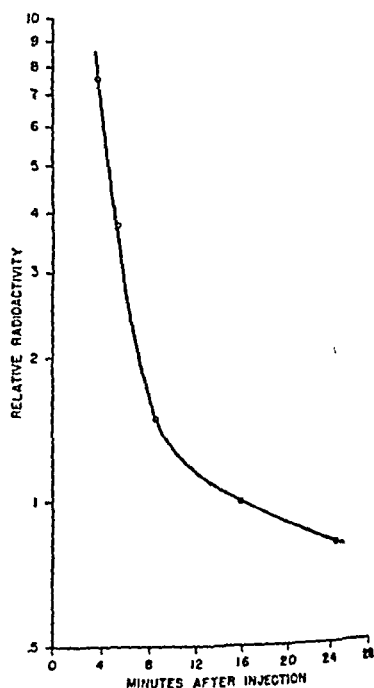


Fig. 5.

Fig. 4.—Disappearance of a large particle gold sol from the circulation of a dog. (Time is measured from the end of the injection period.)

Fig. 5.—Semilogarithmic plot of the data shown in Fig. 4.

Radioactivity measurements were made with the usual thin mica window bell-type Geiger-Müller counter. In the majority of cases the counting tube was connected to a counting-rate meter of the M.I.T. type. In other instances a scaling circuit was employed. Radioactivity was calculated in arbitrary counts per minute per gram of fresh tissue. By determining the activities of tissues at considerable time after autopsy, manganese could be traced in patients who received both elements, since the short-lived gold had then decayed to a negligible level, leaving only the 310-day isotope of manganese

TABLE VII. ANIMAL WEIGHTS, DURATION OF LIFE, AND TYPE OF SOL GIVEN

DOG	WEIGHT (KILOGRAMS)	DURATION OF LIFE AFTER INJECTION	TYPE OF SOL GIVEN
1	8.6	30 min.	Large particle gold sol
2	11.0	30 min.	Small particle gold sol
3	8.0 (Est.)	24 hr.	Large particle gold sol
4	8.0	24 hr.	Small particle gold sol
5	8.0	24 hr.	Manganese dioxide sol
6	9.0	24 hr.	Manganese dioxide sol
7	8.4	48 hr.	Crystalloidal $\text{MnCl}_2$ solution

## DISCUSSION

Drinker and Shaw<sup>5</sup> injected finely divided manganese dioxide intravenously into cats. Their dispersions were prepared by centrifugation and were probably not of uniform particle size. The particles were less than  $1\mu$  in size and were thus small enough to traverse the capillary circulation. The sols were stabilized by adding gum acacia. The disappearance of particles from the circulation was rapid, and in the majority of cases manganese was not found in the blood eighteen minutes after injection. At autopsy 47 per cent of the injected manganese was found in the lung. The content of the liver was 38.3 per cent, of the spleen 4.3 per cent, and of all other organs 10 per cent. Histologic studies indicated that the particles were located within or upon the lining of the capillary endothelium, suggesting that they were not strained out by the capillary bed of the lung by simple filtration. In a separate study Lund, Shaw, and Drinker<sup>6</sup> showed that the cat was unique in this behavior and that rabbits, dogs, guinea pigs, rats, chickens, and turtles concentrated the material predominantly in the liver alone. The content of the spleen was always of the order of 1 or 2 per cent of the initial dose. No separate investigation of the pancreas was conducted.

Since these results suggest that species differences can occur, one could not predict human distribution results with any confidence using our different type of sol prior to the present investigation. The data in Table I are obtained only from single samples of the organs. Only pathologic cases have been studied and absolute percentages of the initial injected dose of manganese in each organ have not been obtained for obvious technical reasons. Nevertheless, the results are similar to those of the previous investigators who use laboratory animals. Thus, we can state with some confidence that the distribution of colloidal manganese in human beings is essentially the same, even when smaller particles are used or smaller amounts of manganese. We hesitate to attach any significance to the lower percentage found in the livers of the two dogs compared to those of the earlier workers, since in our method of preparation an appreciable non-dispersed crystalloidal fraction may have been present. Previous experiments have indicated that this is rapidly excreted.<sup>2</sup> The relative amounts are in good agreement with the earlier work.

The high concentration of radioactivity in the pancreas is of great interest. Examination of the data presented by Reiman and Minot<sup>7</sup> shows that the normal human pancreas contains a relatively high concentration of manganese, being

TABLE VIII. DISTRIBUTION OF CRYSTALLOIDAL MANGANESE IN ONE ANIMAL (Dog 7)

ORGAN	COUNTS PER MINUTE PER GRAM	RATIO OF CONCENTRATION TO HEART	PER CENT DOSE
Pancreas	120	7.5	0.02
Liver	180	11.0	1.50
Spleen	19	1.2	0.005
Heart	16	1.0	-
Gall bladder bile	270	17.0	-

exceeded only by the liver. Since the pancreas does not concentrate colloidal gold it is suspected that the manganese in the pancreas was derived, at least in part, from ionic manganese. In a single exploratory experiment an injection of ionic manganous chloride tagged with  $Mn^{54}$  was given to a dog. The distribution of tagged manganese in liver (multiple samples), spleen and pancreas (entire organs), and heart is shown in Table VIII. The gall bladder contents were rich in tagged manganese as might be expected from the known biliary excretion of the crystalloidal element. Although this experiment does not prove that all of the manganese in the pancreas was derived from ionic manganese, the high pancreatic concentration is suggestive. A complete understanding of the pancreatic concentration must await experiments with dialyzed colloids and further studies of the metabolism of ionic manganese. It is possible that manganese may play a more significant role in pancreatic metabolism than previously has been realized.

It was noted in Table I that the patients lived for variable intervals after the last dose. This dose in nearly all cases contributed almost entirely to the radioactivity at autopsy. There appears to be no really significant change in relative concentration of radioactivity as the elapsed time following the last injection increases. Nevertheless, the values are subject to rather large variations which may obscure small effects.

The doses which were administered were always small. In the case of gold less than 3 mg. were given to a typical patient. In the case of manganese the maximum dose was 3.8 mg. of metallic manganese equivalent (Patient 9), and the majority of doses varied between 0.3 and 1 milligram. Here again the data do not give any picture of variation of distribution with the size of the dose.

It is shown in Fig. 2 that colloidal gold disappears rapidly from the human circulation and that under proper conditions the semilogarithmic plot (Fig. 3) is strictly linear for a considerable period of time. The appreciable time for injection (two or three minutes) plus the short interval before the first blood sample was obtained (one and one-half minutes) is evidently ample to insure circulatory mixing. The exponential decay with a "half-life" of two and one-half minutes suggests that the rate of phagocytosis is strictly proportional to the concentration of colloidal particles in circulation. The disappearance curve for the dog (Figs. 4 and 5) indicates a correspondingly rapid decline. The nonlinearity of the disappearance curve suggests that the dog was not in a uniform circulatory state. Since the animal was not trained and struggled frequently during the experiment, this was not surprising.

The distribution of colloidal gold in different samples of liver and spleen in Dog 4 indicates a systematic variation (Table IV). The activity of equivalent central samples of each lobe is essentially the same, but a systematic increase in concentration is indicated between the peripheral and central portion of the right central lobe in which multiple samples were taken. Similarly, there was a significant variation in the concentration of gold in the spleen samples. Bearing this in mind, it is not difficult to understand the definite variations in comparative results in all cases where determinations were made on single samples of tissue. In spite of such variations, however, the picture is consistent in that liver and spleen are roughly equivalent in concentration of radioactivity and outstandingly high compared to all other tissues. This distribution is very similar to that found by Jones, Wrobel, and Lyons<sup>8</sup> in a single dog which received an intravenous injection of colloidal chromic phosphate containing particles  $1\ \mu$  or less in size.

The different splenic concentrations for gold and manganese is noteworthy indeed. If we are concerned with true phagocytosis, there must be a characteristic difference between the phagocytic action of the spleen for different chemical substances, such that the affinity of the spleen for manganese dioxide is relatively less than for gold. It is of interest that Voigt<sup>9</sup> observed a relatively lower concentration of colloidal silver in the spleen of the rabbit.

Although only one intraperitoneal injection was given, it is of considerable interest that the distribution is roughly similar to that obtained by intravenous injection. Additional research is required before definite conclusions can be established in this matter.

#### SUMMARY

A study of the relative distribution of colloidal particles of manganese dioxide containing radioactive  $\text{Mn}^{52}$  has shown that in the human being liver concentration is high. The concentration of the pancreas and kidney is intermediate and that of the spleen and other organs is low. Two dogs showed a similar picture for liver, pancreas, spleen, and heart, which were the only organs studied. Studies in human subjects and dogs of the distribution of colloidal gold containing radioactive  $\text{Au}^{198}$  showed a high concentration in liver and spleen, intermediate to low in the kidney, and a low concentration in other organs. The difference in the splenic concentration of gold and manganese is striking and suggests a fundamental difference of phagocytic activity for the two substances. The high pancreatic concentration of manganese is of especial significance.

We wish to thank the various members of the Department of Pathology at Vanderbilt University School of Medicine who helped in obtaining autopsy specimens. The assistance of many other staff members also is gratefully acknowledged.

#### REFERENCES

1. Hahn, P. F., and Sheppard, C. W.: Selective Radiation Obtained by the Intravenous Administration of Colloidal Radioactive Isotopes in Diseases of the Lymphoid System, *South. M. J.* 39: 558-562, 1946.
2. Sheppard, C. W., and Hahn, P. F.: Retention and Excretion of Manganese Dioxide Dispersions Administered Intravenously to Humans, *South. M. J.* 39: 562-565, 1946.

3. Ellenburger, W., and Baum, H.: *Anatomic des Hundes*, Berlin, 1891, Paul Parey.
4. Hahn, P. F.: *Radioactive Iron Procedures: Purification, Electroplating, and Analysis*, *Indust. & Engin. Chem. (Anal. Ed.)* 17: 45-46, 1945.
5. Drinker, C. K., and Shaw, L. A.: *Quantitative Distribution of Particulate Material (Manganese Dioxide) Administered Intravenously to the Cat*, *J. Exper. Med.* 33: pages 77-98, 1921.
6. Lund, C. C., Shaw, L. A., and Drinker, C. K.: *Quantitative Distribution of Particulate Material (Manganese Dioxide) Administered Intravenously to the Dog, Rabbit, Guinea Pig, Rat, Chicken, and Turtle*, *J. Exper. Med.* 33: 231-238, 1921.
7. Reiman, C. K., and Minot, A. S.: *A Method for Manganese Quantitation in Biological Material Together With Data on the Manganese Content of Human Blood and Tissues*, *J. Biol. Chem.* 42: 329-345, 1920.
8. Jones, H. B., Wrobel, C. J., and Lyons, W. R.: *A Method of Determining the Distribution to the Reticulo-Endothelial System and Adjacent Tissues*, *J. Biol. Chem.* 153: 783-788, 1944.
9. Voigt, J.: *Ueber die Verteilung und das Schicksal des Kolloidalen Silbers im Säugetierkörper*, *Biochem. Ztschr.* 63: 409-424, 1914.

# EFFECT OF PREGNANCY ON THE COMPLEMENT OF GUINEA PIGS

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ALTHOUGH it is generally known among serologists that the serum of a pregnant guinea pig is likely to be unsatisfactory for use in complement fixation, no study of the exact relationship between pregnancy and complement in this animal has been found in the literature. The question is of importance as a preliminary step in the study of complement variations in disease, since some knowledge of the possible variations of complement in different physiologic states is necessary for the proper interpretation of data obtained from a group of experimental animals. The present study was undertaken in an attempt to delineate the range of variation of complement in pregnant guinea pigs.

## METHODS

*Selection and Care of the Animals.*—A group of nine healthy adult guinea pigs, known to be nonpregnant, was selected as a control in the experiment. Members of the group ranged in age from 10 to 14 months and in weight from 450 to 700 grams.

The group of pregnant guinea pigs was composed of females recently bred or known to be pregnant. The group was screened to exclude any animals that did not correspond approximately to the control group in respect to age, size, and general health. Various stages of pregnancy were represented in the group, particularly the later stages. A total of fourteen animals was observed both during and after parturition. Two additional animals were acquired after they had given birth and were observed only during the postparturition period.

All animals were maintained separately in clean, dry cages. They were fed a controlled diet of oats, Rockland guinea pig Pellets, dried alfalfa, and fresh cabbage. No water was offered to the animals at any time.

The time of parturition of each pregnant guinea pig was recorded from actual observation or from an estimate based on the appearance of the litter after birth had occurred.

*Method of Obtaining Blood Samples.*—A blood sample of 0.4 to 0.6 c.c. was withdrawn twice weekly from each guinea pig by bleeding from a small incision in the marginal vein of the ear. This method appears to have no undesirable effects upon the animal and, for that reason, is to be preferred to the more common method of cardiac puncture, particularly in a study of this type.

The blood sample was collected in a clean 3 c.c. serologic test tube, covered with a small piece of parafilm to protect the sample and retard evaporation,

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allowed to clot for twenty minutes at 2 to 4° C., and then centrifuged at 1,600 r.p.m. for fifteen minutes. The supernatant serum was decanted carefully into another clean 3 c.c. test tube and stored at 2 to 4° C. until used. In all cases the serum was titrated within two hours after collection of the sample.

*Method of Titration.*—The assay of complement was performed by a method independently devised in this laboratory. Similar methods have been used by Mayer, Eaton, and Heidelberger<sup>1</sup> and by Kent, Bukantz, and Rein.<sup>2</sup>

The serum to be tested was diluted 1:100 in 0.9 per cent saline solution. Immediately after dilution, portions of the diluted serum were pipetted into each of three clear glass test tubes (of the type designed for use in the Klett-Summerson photoelectric colorimeter). For sera of a high titer, the portions used were 1.00, 1.50, and 2.00 c.c., respectively. For sera of low titer, portions of 1.50, 2.00, and 2.50 c.c. were used. Varying the quantities in this fashion provided an optimal amount of serum in at least two of the three tubes. The volume in each tube was adjusted to 2.50 c.c. by the addition of saline solution.

To each tube 2.50 c.c. of a suspension of sensitized sheep red cells (prepared as herein described) were then added. The tubes were shaken thoroughly and incubated for thirty minutes at 37° Centigrade. After the incubation period, the tubes were centrifuged at 1,600 r.p.m. for five minutes.

The color of the supernatant fluid in each tube was measured with a Klett-Summerson photoelectric colorimeter equipped with a standard green filter over the light source. The scale of the instrument is calibrated in units which are proportional to the concentration of dissolved hemoglobin in the supernatant layer. Thus, the per cent hemolysis occurring in a given tube may be computed as the ratio of the color reading to that of the supernatant layer in a completely hemolyzed sample. The latter value was determined for each set of titrations by taking the color reading of 2.50 c.c. of the sensitized cell suspension laked in 2.50 c.c. of distilled water.

*Preparation of the Standard Sheep Cell Suspension.*—One volume of freshly drawn packed sheep erythrocytes was washed four times in 10 volumes of saline solution. The washed, packed cells were suspended in saline solution in sufficient amount so that a sample of this suspension (diluted 1:20 in saline solution) gave a color reading of 280 on the colorimeter. When thus adjusted in concentration, the cell suspension was then diluted with an equal volume of saline solution containing 5 units of rabbit antish sheep hemolysin of a titer of at least 1:4,000. After mixture of the cells and hemolysin, the suspension was allowed to stand for thirty minutes at 2 to 4° C. before use. The final suspension contained about 2 per cent (by volume) of packed cells and approximately  $2.5 \times 10^8$  erythrocytes per cubic centimeter.

*Calculation of Results.*—The amount of 1:100 serum required to effect 50 per cent hemolysis was obtained from a form of the von Krogh equation (3):

$$x = k \left( \frac{y}{1 - y} \right)^{1/n}$$

Where  $\bar{x}$  equals amount of hemolytic serum,  $y$  equals fraction of total cells hemolyzed, and  $k$  and  $1/n$  are constants for a given set of experimental conditions. When expressed in the logarithmic form and adapted to the case of 50 per cent hemolysis, the equation may be written as:

$$x_2 = \text{antilog} \left( \log x_1 - \frac{1}{n} \log \frac{Y}{100 - Y} \right)$$

Where  $x_2$  equals cubic centimeters of 1:100 serum required for 50 per cent hemolysis,  $x_1$  equals cubic centimeters of 1:100 serum actually used, and  $Y$  equals per cent hemolysis actually obtained. For the titration used, the value

of the constant  $1/n$  was found to be  $0.22 \pm .02$ . Curves of  $x_2$  versus  $\frac{Y}{100 - Y}$

were plotted on log-log graph paper for the various discrete values of  $x_1$ . From the resulting straight-line nomograph, the value of  $x_2$  for any values of  $x_1$  and  $Y$  could be determined by interpolation.

The von Krogh equation is a satisfactory empirical statement of the relationship only when the hemolysis is between 10 and 90 per cent. The serum portions ( $x_1$ ) have been chosen so that the resulting hemolysis will ordinarily be within this range. In this experiment the value of  $x_2$  was taken as the average found in the tubes (usually all three) in which more than 10 but less than 90 per cent hemolysis occurred.

The *complement activity* was defined arbitrarily as the number of 50 per cent hemolytic units per cubic centimeter of serum. In terms of  $x_2$  this is expressed as:

$$\text{Complement activity} = \frac{100}{x_2}$$

Complement activity bears a direct relationship to the concentration of complement in the serum and provides a convenient figure for graphing and tabulation.

#### RESULTS AND DISCUSSION

The results of 262 separate complement titrations are shown as a "scatter diagram" in Fig. 1. Complement activity is depicted as a function of time in days relative to the date of parturition. For the nonpregnant guinea pigs, the distribution of points along the time axis was in terms of the number of days before or after the middle of the observation period.

Inspection of the graph apparently discloses that (1) the pregnant guinea pigs had substantially lower complement activities than the nonpregnant ones and (2) the postparturition period in the group of pregnant guinea pigs was marked by a gradual increase in complement activity.

These observations may be further substantiated by statistical analysis.\* The average complement activity for each nonpregnant guinea pig is shown

\*The authors are indebted to Dr. Huldah Bancroft, Department of Preventive Medicine, Medical School, Western Reserve University, for assistance in the statistical evaluation.



TABLE I. COMPLEMENT ACTIVITY IN NONPREGNANT GUINEA PIGS

	GUINEA PIG										GROUP MEAN
	1	9	10	24	35	36	44	45	46		
Days in observation period	37	37	45	42	37	37	16	16	16	--	
Number of separate titrations	11	11	14	13	10	11	5	5	5	--	
Average C' activity	80	84	92	67	59	84	74	62	68	74	
Highest C' activity	100	122	126	86	80	110	84	82	87	--	
Lowest C' activity	52	55	67	55	44	61	59	47	49	--	

in Table I; the average complement activities for the pregnant animals, both before and after parturition, are shown in Table II. The animals were divided into three groups: nonpregnant, ante partum, and post partum. The statistical significance of the differences among the means of complement activity in the three groups was determined by the method devised by Fisher<sup>4</sup> for groups small in number.

The results of the statistical analysis are shown in Table III. The value of "t" in the tests of significance are well above the minimum values required to demonstrate significant differences among the three groups.

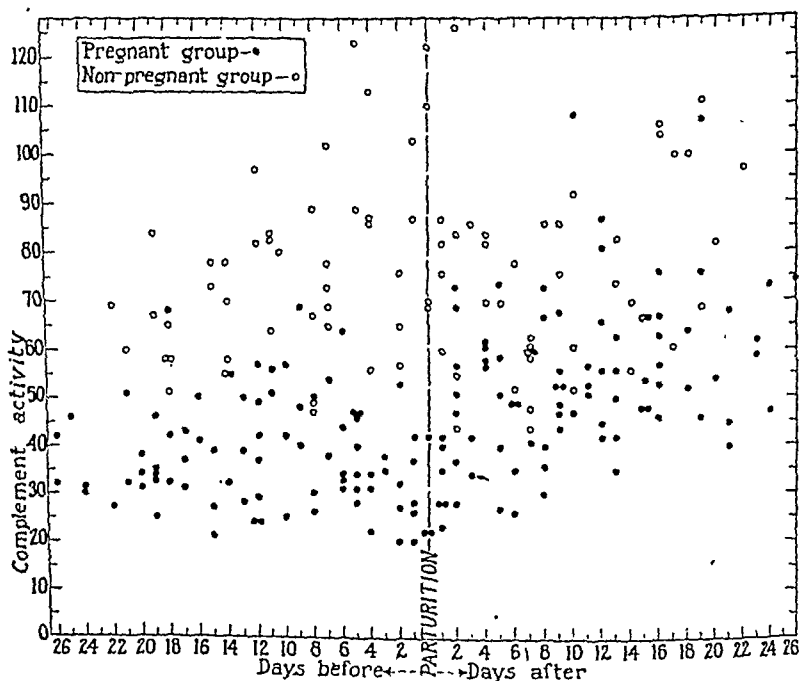


Fig. 1.—Scatter diagram of complement activity before and after parturition. Points for the nonpregnant group are located according to the number of days before or after the middle day of the observation period.

It should be pointed out that, although a significant difference in complement activity between the nonpregnant and post-partum groups exists, the gradual upward trend in the post-partum group predicts that most of these animals would have returned to normal within five weeks after parturition. This prediction is confirmed by the frequent observation of the authors that the serum

TABLE II. COMPLEMENT ACTIVITY IN PREGNANT GUINEA PIGS BEFORE AND AFTER PARTURITION

	GUINEA PIG																GROUP MEAN
	21	25	26	27	28	29	30	31	32	33	34	37	38	41	42	43	
<i>Before Parturition</i>																	
Days in observation period	21	19	7	22	3	31	35	25	19	25	13	20	27	0	0	12	--
Number of separate titrations	7	6	2	7	2	10	11	7	6	7	4	11	8	0	0	3	--
Average C' activity	43	34	30	31	21	32	47	43	27	36	31	45	49	-	-	33	36
Highest C' activity	53	51	33	38	22	42	69	64	34	44	47	57	68	-	-	35	--
Lowest C' activity	37	22	27	25	20	20	32	30	21	22	24	35	28	-	-	29	--
<i>After Parturition</i>																	
Days in observation period	19	16	26	18	30	15	11	12	13	12	24	17	10	25	25	3	--
Number of separate titrations	6	5	8	6	8	5	4	4	5	4	7	5	3	7	7	2	--
Average C' activity	62	44	67	44	48	54	57	58	57	51	49	57	79	55	40	42	54
Highest C' activity	76	50	107	61	74	67	67	81	92	66	73	76	108	68	48	42	--
Lowest C' activity	44	34	37	23	26	47	51	35	28	28	35	40	60	40	27	42	--

TABLE III. STATISTICAL EVALUATION OF DIFFERENCES AMONG NONPREGNANT, ANTE-PARTUM, AND POST-PARTUM GROUPS OF GUINEA PIGS

GROUPS COMPARED	FIRST MEAN ( $\bar{x}_1$ )	NO. ( $n_1$ )	SECOND MEAN ( $\bar{x}_2$ )	NO. ( $n_2$ )	S.D. OF DIFFER- ENCE ( $s^2$ ) *	DEGREES OF FREEDOM	t†	MINIMUM VALUE OF t FOR P = 0.01
Nonpregnant group and Ante-partum group	74	9	36	14	90.2	21	9.4	2.831
Ante-partum group and Post-partum group	36	14	54	16	82.0	28	6.2	2.763
Nonpregnant group and Post-partum group	74	9	54	16	106	23	4.7	2.797

\*Standard deviation of difference =  $s^2 = \frac{\sum (x_1 - \bar{x}_1)^2}{n_1} + \frac{\sum (x_2 - \bar{x}_2)^2}{n_2} \div n_1 + n_2 - 2$

$$\dagger t = \frac{|\bar{x}_1 - \bar{x}_2|}{s} \sqrt{\frac{n_1 n_2}{n_1 + n_2}}$$

of a female guinea pig four to six weeks after parturition is fully as active in fixation and hemolysis as the sera of adult virgin female guinea pigs kept under the same dietary conditions.

#### CONCLUSIONS

1. Pregnancy in the guinea pig is accompanied by a substantial decrease in complement activity of the serum.
2. After parturition complement activity gradually increases toward the level normal for nonpregnant female guinea pigs.

#### REFERENCES

1. Mayer, M. M., Eaton, B. B., and Heidelberger, M.: Spectrophotometric Standardization of Complement for Fixation Tests, *J. Immunol.* 53: 31, 1946.
2. Kent, J. F., Bukantz, S. C., and Rein, C. R.: Studies in Complement Fixation. I. Spectrophotometric Titration of Complement; Construction of Graphs for the Direct Determination of the 50% Hemolytic Unit, *J. Immunol.* 53: 37, 1946.
3. von Krogh, M.: Colloidal Chemistry and Immunology, *J. Infect. Dis.* 19: 452, 1916.
4. Fisher, R. A.: Statistical Methods for Research Workers, ed. 10, Edinburgh, 1946, Oliver & Boyd, Ltd.

TOXICITY STUDIES OF  $\alpha$  (4-HYDROXY-3,5-DIIODO-BENZYL-N-BUTYRIC ACID) AND  $\alpha$  (4-HYDROXY-3,5-DIIODO-BENZYL-N-CAPROIC ACID): COMPOUNDS PROPOSED AS GALL BLADDER CONTRAST AGENTS

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**V**ISUALIZATION of the gall bladder through the use of a new series of compounds has been described in a recent report.\* Of these  $\alpha$  (4-hydroxy-3,5-diiodo-benzyl-n-butyric acid) and  $\alpha$  (4-hydroxy-3,5-diiodo-benzyl-n-caproic acid) were selected as having superior properties from the point of view of concentration in the gall bladder with a minimum of reaction of the patient. Since these compounds may be used extensively in clinical work it was deemed advisable to undertake a study of their toxicity.

Both substances appear as fine, white crystalline powders. They are insoluble in water but soluble as the sodium salts. Through the use of sodium hydroxide  $\alpha$  (4-hydroxy-3,5-diiodo-benzyl-n-butyric acid) forms a solution with a pH of 7.5, while under similar conditions  $\alpha$  (4-hydroxy-3,5-diiodo-benzyl-n-caproic acid) forms a solution of pH 8.5. The compounds were purified before use by dissolving them in 5 per cent  $\text{NaHCO}_3$  solution (1 Gm. to 100 c.c. of bicarbonate solution). Charcoal was added and the solution filtered. The acids, precipitated by the addition of concentrated hydrochloric acid, were filtered and, after drying, recrystallized from carbon tetrachloride.

EXPERIMENTAL PROCEDURE

For intravenous administration a 25 per cent solution of each compound was used. For oral use the powder was intimately mixed with small amounts of ground red meat or fish. In all experiments cats of 2.5 to 4 kilograms of body weight were used. These cats were observed for a period of one week before starting the experiments.

During the experiment the weights of each animal were recorded frequently and their general behavior and reaction to the drugs were observed. The cats were then sacrificed at the termination of the period of observation, which varied from two to six weeks. Necropsy studies then were made.

DISCUSSION OF RESULTS

Administration of these drugs to cats by the oral route causes much less evidence of toxic effects than is the case with intravenous administration. With the exception of an impaired appetite for a period of forty-eight hours, the cats showed no untoward reaction to the oral administration of the drugs. Vomiting was not observed at any time and all the animals gained weight. During the period of observation two of the cats delivered normal litters of kittens. At

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\*Epstein, B., Natelson, S., and Kramer, B.: A New Series of Radiopaque Compounds. I. Chemical Structures, Channels of Excretion, and Roentgenographic Uses, *Am. J. Roentgenol.* 50: 201-207, 1946.

TABLE I. EFFECT OF ORAL ADMINISTRATION OF SINGLE DOSE OF  $\alpha$  (4-HYDROXY-3,5-DIODO-BENZYL-N-BUTYRIC ACID)

CAT	DOSE/KG. (MG.)	TOTAL DOSE (GM.)	PERIOD OF OBSERVATION (WEEKS)	REMARKS
22	750	1.60	4	No vomiting; appetite impaired for 48 hours
1	910	3.19	6	No vomiting; appetite impaired for 48 hours
6	940	3.20	6	No vomiting; appetite impaired for 48 hours
2	950	4.09	6	No vomiting; appetite impaired for 48 hours
25	1,000	3.20	4	No vomiting; appetite impaired for 48 hours
18	1,000	2.60	3	No vomiting; appetite impaired for 48 hours
19	1,000	3.10	3	No vomiting; appetite impaired for 48 hours
27	1,000	1.90	3	No vomiting; appetite impaired for 48 hours
26	1,000	1.40	3	No vomiting; appetite impaired for 48 hours
24	1,000	3.50	3	No vomiting; appetite impaired for 48 hours
31	1,000	3.70	3	No vomiting; appetite impaired for 48 hours

TABLE II. EFFECT OF ORAL ADMINISTRATION OF SINGLE DOSE OF  $\alpha$  (4-HYDROXY-3,5-DIODO-BENZYL-N-CAPROIC ACID)

CAT	DOSE/KG. (MG.)	TOTAL DOSE (GM.)	PERIOD OF OBSERVATION (WEEKS)	REMARKS
20	830	3.07	4	No vomiting; appetite impaired for 48 hours
7	835	3.09	6	No vomiting; appetite impaired for 48 hours
30	840	3.02	4	No vomiting; appetite impaired for 48 hours
3	845	2.45	6	No vomiting; appetite impaired for 48 hours
21	885	3.01	4	No vomiting; appetite impaired for 48 hours
5	890	3.20	6	No vomiting; appetite impaired for 48 hours
4	910	3.19	6	No vomiting; appetite impaired for 48 hours
23	1,000	2.50	4	No vomiting; appetite impaired for 48 hours
29	1,000	3.10	3	No vomiting; appetite impaired for 48 hours
28	1,000	2.70	3	No vomiting; appetite impaired for 48 hours

necropsy there were no abnormal findings either in the gross or in histologic sections, except that a minimal degree of cloudy swelling was noted in the liver and kidneys of two cats.

No estimation was made of the lethal dose orally, because all animals survived doses of 1,000 mg. per kilogram of body weight. Higher doses were not studied, for the total dose administered to human beings orally does not exceed 100 mg. per kilogram of body weight.

TABLE III. EFFECT OF INTRAVENOUS ADMINISTRATION OF SINGLE DOSE OF  $\alpha$  (4-HYDROXY-3,5-DIODO-BENZYL-N-BUTYRIC ACID). TO UNANESTHETIZED CATS

CAT	DOSE/KG. (MG.)	TOTAL DOSE (MG.)	PERIOD OF OBSERVATION (WEEKS)	REMARKS
2a	175	810	6	No untoward reaction
27a	175	330	6	No untoward reaction
3a	180	540	6	Salivated; slight ataxia completely recovered in 30 minutes
4a	260	1,090	6	No untoward reaction
28a	300	810	6	No untoward reaction
5a	310	1,080	6	Drowsy; occasional jerky movements; deep respirations
1a	350	1,240	6	Salivated; no other reaction
19a	350	1,085	6	Rapid respirations; recovered in 1 hour
7a	380	1,350	6	Salivated; occasional jerky movements; recovered in 45 minutes
18a	400	1,040	6	Rapid respirations; coma; recovered after several hours
30a	500	1,850	-	Rapid breathing; coma; death after 45 minutes

TABLE IV. EFFECT OF INTRAVENOUS ADMINISTRATION OF SINGLE DOSE OF  $\alpha$  (4-HYDROXY-3,5-DIODO-BENZYL-N-CAPROIC ACID) TO UNANESTHETIZED CATS

CAT	DOSE/KG. (MG.)	TOTAL DOSE (MG.)	PERIOD OF OBSERVATION (WEEKS)	REMARKS
14a	227	750	6	Rapid respirations; coma; twitchings; fully recovered in 24 hours
13a	240	750	6	Rapid respirations; coma; twitchings; fully recovered in 24 hours
9a	275	1,275	6	No reaction; took feeding 1 hour later
16a	275	625	4	Rapid breathing; twitchings during 30 minutes
12a	290	875	4	Mild convulsions; rapid breathing; recovered in 1 hour
8a	305	1,325	6	Convulsions; rapid breathing; coma; recovered in 24 hours
10a	315	1,025	4	Convulsions lasting for about 1 hour; coma; recovered after 24 hours
11a	335	1,250	6	Rapid respirations; coma; twitchings; fully recovered in 24 hours
15a	375	750	-	Violent convulsions; coma; died after 15 minutes

Study of  $\alpha$  (4-hydroxy-3,5-diiodo-benzyl-n-butyric acid), in intravenous doses below 200 mg. per kilogram, failed to demonstrate any abnormal reactions. Above this level the administration of the drug caused convulsions, rapid respiration, and varying degrees of drowsiness; such reactions were more marked above 300 mg. per kilogram. When 400 mg. per kilogram were administered intravenously the cats became comatose, and at 500 mg. per kilogram death followed after a coma of forty-five minutes' duration. The lethal intravenous dose of this drug is of the order of 400 to 500 mg. per kilogram.

Following the intravenous studies of  $\alpha$  (4-hydroxy-3,5-diiodo-benzyl-n-butyric acid), the intravenous studies of  $\alpha$  (hydroxy-3,5-diiodo-benzyl-n-caproic acid)

were started at a level exceeding 200 mg. per kilogram of body weight. At levels of 200 to 275 mg. per kilogram rapid respiration was noted, together with some ataxia and twitchings of the extremities. At 300 mg. per kilogram convulsions were observed, and at 315 mg. per kilogram the convulsions were marked, with rapid breathing. This rapid breathing persisted for several hours. When the dose was raised to 375 mg. per kilogram death of the injected animal occurred after fifteen minutes.

The animals that survived the intravenous administration of the drugs recovered completely after twenty-four to forty-eight hours. They took their feeding well thereafter and gained weight. When the animals were sacrificed at the end of two to four weeks no abnormal findings were observed on gross examination of the liver and kidneys.

In the two animals that died following the administration of the lethal doses, petechial hemorrhages were noted in the lungs. The livers and kidneys showed moderate degrees of cloudy swelling.

#### SUMMARY

1. The toxic effects of  $\alpha$  (4-hydroxy-3,5-diiodo-benzyl-n-butyric acid) and  $\alpha$  (4-hydroxy-3,5-diiodo-benzyl-n-caproic acid) were studied, both by oral and by intravenous administration, in a total of forty-two cats.

2. Oral administrations of the drug were tolerated in doses up to 1,000 mg. per kilogram, without vomiting. Appetite was impaired for forty-eight hours.

3. The intravenous administration of  $\alpha$  (4-hydroxy-3,5-diiodo-benzyl-n-butyric acid) caused convulsions in doses higher than 200 mg. per kilogram and death at 500 mg. per kilogram. Intravenous administration of  $\alpha$  (4-hydroxy-3,5-diiodo-benzyl-n-caproic acid) caused convulsions at 200 mg. per kilogram and death at 375 mg. per kilogram.

4. All the animals given sublethal doses of the drugs recovered completely and gained weight at the end of two to four weeks.

5. Necropsy studies revealed no abnormal findings, except in cats which were given lethal doses of the drugs. In these cats petechiae in the lungs and cloudy swelling in the liver and kidneys were found.

## LABORATORY METHODS

### THE MICROCOLORIMÉTRIC ESTIMATION OF PLASMA PROTEINS

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IN A recent publication<sup>1</sup> we described a colorimetric method utilizing the Sakaguchi reaction for the estimation of total proteins and protein fractions in human plasma, nephrotic urine, and abdominal fluid in terms of arginine which was shown to have an accuracy comparable to the micro-Kjeldahl method. Subsequent experiments have shown that because of the relatively negligible nonprotein arginine content of the blood greater economy of time and blood sample without appreciable loss of accuracy can be achieved by: (1) determination of total plasma proteins on a 1 c.c. aliquot of a sample prepared by diluting 0.1 c.c. of plasma to 2 c.c. volume with 10 per cent NaOH instead of an alkaline solution of the trichloroacetic acid precipitated proteins, (2) estimation of albumin and globulin by direct measurement of both proteins as arginine in fraction derived from the same 0.2 c.c. sample of plasma rather than by difference of total protein and globulin, and (3) elimination of the three-hour incubation period usually employed in the separation of albumin and globulin fractions of the plasma. The rapidity and ease of operation which has been gained by these modifications would seem to recommend the method for routine clinical work.

#### EXPERIMENTAL

##### *Reagents.*—

*Sodium Hypochlorite:* A 0.06 N solution was prepared as needed from the commercially available Clorox. The necessary dilution of the stock product was ascertained iodometrically as follows: To 1 c.c. of Clorox in a 125 c.c. Erlenmeyer flask are added 25 c.c. of chlorine-free water in which has been dissolved 1 Gm. of potassium iodide. The mixture was then titrated with 0.1 N sodium thiosulfate, 1 c.c. of starch indicator being used. The stock product has been found to be fairly stable for three to four months if kept in the refrigerator in a brown bottle.

*Sodium Hydroxide:* A 10 per cent solution.

*Urea:* A 20 per cent solution.

*$\alpha$ -Naphthol:* One hundred milligrams of the resublimed product are dissolved in 100 c.c. of 95 per cent ethanol. The solution is kept in a brown bottle and stored in the refrigerator.

*Arginine Standard:* One (plus) arginine hydrochloride\* (26.6 per cent N found), 12.05 mg., is weighed accurately and dissolved in 100 c.c. of a saturated aqueous solution of benzoic acid. One cubic centimeter of this solution is equivalent to 100  $\gamma$  of the free base. This solution is stored in the refrigerator.

*Ether:* Reagent grade can be used as obtained.

*Sodium Sulfate, Anhydrous:* A 22 per cent solution is employed and is stored in a 37° C. incubator.

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*Analytic Procedures.—*

**Total Plasma Proteins:** One-tenth cubic centimeter of plasma (derived from blood collected over lithium oxalate) in a graduated 15 c.c. conical centrifuge tube was made to the 2 c.c. mark with 10 per cent NaOH. A 1 c.c. aliquot of the thoroughly mixed sample was transferred to a graduated colorimeter tube, and 5 c.c. of water and 1 c.c. of alcoholic naphthol were added in rapid succession. After standing for five minutes, 1 c.c. of sodium hypochlorite is added, followed exactly one minute later by the addition of 2 c.c. of 20 per cent urea solution. The resulting solutions are mixed by inverting the tubes and read in the Klett-Summers colorimeter with Filter S-54 after a lapse of five minutes. The color intensity of the test mixture has been found to remain constant for an hour or more after the initial reaction period, so that there is no need to hasten the color measurement. A parallel determination is also done on a 1 c.c. aliquot of the standard and a reagent blank.

**Plasma Albumin and Globulin:** To 0.2 c.c. of plasma in a 50 c.c. round bottom, small neck centrifuge tube (A. H. Thomas No. 3123) was added with mixing 7 c.c. of 22 per cent sodium sulfate and then 3 c.c. of ethyl ether.<sup>2</sup> This mixture was shaken vigorously for two minutes and then centrifuged at 3,000 r.p.m. for ten minutes. The globulin which collected at the ether-water interface was separated from both liquid layers by careful decantation into a 25 c.c. graduated cylinder. The volume of the aqueous, albumin-containing fraction was adjusted to 10 c.c. by the addition of water, and a 5 c.c. aliquot of this layer is removed by pipette to a 10 c.c. graduated colorimeter tube. Subsequent to the addition of 1 c.c. of 10 per cent NaOH, the protein content is determined in terms of arginine described as follows.

The disk of precipitated globulin was dissolved in 2 c.c. of 10 per cent NaOH and then 2 c.c. of water were added. Gentle heating of the mixture in a hot water bath drives off the residual ether and thereby greatly facilitates solution of the protein. A 2 c.c. aliquot of the cooled sample was transferred to a colorimeter tube and submitted to the Sakaguchi reaction after the addition of 4 c.c. of water.

The alkaline dilution of the whole or fractionated plasma of specimens containing 400 mg. per cent of cholesterol or more should be shaken with 5 c.c. of ether prior to removal of sample for the arginine determination.

Attention is called to the fact that in all revisions of sample size which different circumstances may necessitate, it is imperative that the sample be contained in a 6 c.c. volume which shall include the equivalent of 1 c.c. of 10 per cent NaOH prior to the addition of the other reagents.

**Calculations:** After the colorimeter is set to zero for the reagent blank, the arginine present in the protein samples is estimated by comparison with the value of the arginine standard. The protein equivalent is obtained by multiplying the arginine values by the dilution factors and 19.2, a factor derived from the mean arginine content of human plasma albumin and globulin.<sup>3</sup>

## RESULTS

The possibility of the present modification of our previously described method for the determination of plasma proteins was impressed upon us by the finding that protein-free filtrates obtained from 5 c.c. samples of ten different human plasma gave negative Sakaguchi tests. This observation indicated that failure to separate the proteins from whole plasma would not introduce any appreciable errors in the final values.

Our experiments amply confirmed the observations of Kingsley<sup>2</sup> that when ether is employed in conjunction with sodium sulfate no preliminary incubation period is required for the separation of globulin and albumin. It has also been found that serum or plasma derived by the use of other oxalates or other anti-coagulants can be used with this method.

The total plasma values obtained by the usual micro-Kjeldahl method and three variants of the arginine method are listed in Table I. It will be noted



TABLE I. COMPARISON OF TOTAL PLASMA PROTEIN VALUES OBTAINED BY VARIOUS TECHNIQUES

SPECIMENS	MICRO-KJELDAHL METHOD, PROTEIN = $N \times 6.25$ (GM. %)	ARGININE METHOD, PROTEIN = ARGININE $\times 19.2$		
		TRICHLOROACETIC ACID-PRECIPITATED PROTEIN (GM. %)	PLASMA DILUTION (GM. %)	TOTAL PLASMA PROTEIN = ALBUMIN + GLOBULIN (GM. %)
Ka, normal, 2 years	7.65	7.70	7.66	7.73
Ec, nephrotic, 1.5 years	3.80	3.84	3.80	3.93
As, nephrotic, 2 years	5.75	5.74	5.78	5.78
Sa, nephrotic, 3 years	4.48	4.46	4.57	4.58

that the figures obtained by the two techniques described in the present report, dilution of the whole plasma and addition of the individually determined albumin and globulin values, are in good accord with those obtained with the arginine method utilizing the trichloroacetic acid-precipitated plasma proteins as well as those of the micro-Kjeldahl procedure.

## COMMENT

The accuracy, speed of operation, and economy of sample achieved by the present modification of the arginine method for the determination of plasma proteins would seem to favor its adoption for routine or clinical studies. Although these procedures are somewhat more involved and time consuming than the specific gravity methods,<sup>4</sup> the higher degree of accuracy which they afford makes the additional effort which they entail seem worth while. This is especially true of the copper sulfate method which has recently been shown by Adams and Ballou<sup>5</sup> to exhibit such poor correlation between the specific gravity measurement and protein content as determined by the micro-Kjeldahl procedure as to make it valueless as a measure of total serum proteins of the blood. This and similar findings reported by Looney<sup>6</sup> and Zozaya,<sup>7</sup> however, have been contradicted by the observations of Atchley and co-workers.<sup>8</sup> The biuret method of Kingsley<sup>2</sup> which is also rapid and employs only small amounts of plasma proved unsatisfactory in our hands. Our difficulties arose from (1) lack of stability of the alkaline copper sulfate reagent, (2) development of uncontrollable turbidity in test samples, especially with specimens from nephrotic patients, and (3) lack of consistent correlation of biuret plasma or serum values with micro-Kjeldahl figures.

Attention is called to the fact that in physiologic or pathologic hyperaminoacidemia, which would naturally tend to increase the amount of free circulating arginine, the plasma or serum proteins should be precipitated with trichloroacetic acid prior to the application of the Sakaguchi reaction as previously reported,<sup>1</sup> rather than employ the procedure described here.

## SUMMARY

A rapid microcolorimetric method for the determination of total plasma proteins or plasma albumin and globulin has been described which yields values comparable to those obtained by the micro-Kjeldahl procedure.

## REFERENCES

1. Albanese, A. A., Irby, V., and Saur, B.: The Colorimetric Estimation of Proteins in Various Body Fluids, *J. Biol. Chem.* 166: 231, 1946.
2. Kingsley, G. R.: The Determination of Serum Total Protein, Albumin and Globulin by the Biuret Reaction, *J. Biol. Chem.* 131: 197, 1939.  
Kingsley, G. R.: A Rapid Method for the Separation of Serum Albumin and Globulin, *J. Biol. Chem.* 133: 731, 1940.  
Kingsley, G. R.: The Direct Biuret Method for the Determination of Serum Proteins as Applied to Photoelectric and Visual Colorimetry, *J. LAB. & CLIN. MED.* 27: 840, 1942.
3. Block, R. J., and Bolling, D.: The Amino Acid Composition of Proteins and Foods, Springfield, Ill., 1945, Charles C Thomas, Publisher, pp. 58-60.
4. Kagan, B. M.: A Simple Method for the Estimation of Total Protein Content of Plasma and Serum. II. The Estimation of Total Protein Content of Human Plasma and Serum by the Use of the Falling drop Method, *J. Clin. Investigation* 17: 373, 1938.  
Phillips, R. A., Van Slyke, D. D., Dale, V. P., Emerson, K., Jr., Hamilton, P. B., and Archibald, R. M.: Copper Sulphate Method for Measuring Specific Gravities of Whole Blood and Plasma. From the United States Navy Research Unit at the Hospital of the Rockefeller Institute for Medical Research. Commanding Officer, Commander Thomas M. Rivers.
5. Adams, M. A., and Ballou, A. N.: A Comparison Between the Values for Plasma or Serum Protein as Obtained by the Specific Gravity and the Micro-Kjeldahl Methods, *J. LAB. & CLIN. MED.* 31: 507, 1946.
6. Looney, J. M.: The Relation Between Specific Gravity of Blood Serum and Its Protein Concentration, *J. LAB. & CLIN. MED.* 27: 1463, 1942.
7. Zozaya, J.: A Physicochemical Study of Blood Sera, *J. Biol. Chem.* 110: 599, 1935.
8. Atchley, J., Bacon, R., Curran, G., and David, K.: A Clinical Evaluation of the Copper Sulfate Method of Measuring Specific Gravities of Whole Blood and Plasma, *J. LAB. & CLIN. MED.* 30: 830, 1945.

## A PULSATING PERFUSION APPARATUS

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THIS apparatus, announced in *Science*,<sup>2a</sup> is a considerable improvement over the one described in 1939,<sup>1</sup> differing in form and size, embodying some entirely new additions, yet retaining the essential features of construction of the older apparatus. Its general appearance is shown in Fig. 1, and details of construction are shown in Figs. 2 to 6. It is so planned that it can operate on a small quantity of blood and that the organ can be studied under a microscope during perfusion. With the apparatus and its specimen chamber shown in Fig. 1, the stomach, pancreas, and part of the duodenum of the rat can be perfused with whole rat blood.

In Figs. 2 to 6 only the essentials are shown. The chief portion of the apparatus consists of a number of pieces of  $\frac{1}{8}$  inch plate glass, the largest (Plates I and II) measuring  $4\frac{3}{8}$  by 7 inches, held together by twelve bolts and milled nuts. The latter are not shown in the drawings given, but in Fig. 5 their positions are indicated. Most of the other essential parts are fabricated from glass tubing and are attached to the plates by ground taper joints.

The channels or "vessels" through which the blood or other fluid circulates consist of small holes drilled through the plates and half-round grooves cut in one plate and covered by another. Most of these grooves are on the under side of the second plate (II) from the bottom; some are in other plates as shown in the drawings. The arrangement or plan of these channels and their relations to the reservoir and pump and other parts are shown in Fig. 5. Some appear in section in Fig. 6.

The essential parts of the pump are two valve chambers, two valves, the three rubber tubes and bulbs, connecting channels, and the electromagnet and mechanism for compressing the middle tube (No. 2). The valve chambers are large holes cut in the middle plate (IV) of the three making up the pump. The valves are small triangular disks of glass (Figs. 3, 5, 6), one of which rests on the openings in the bottom of each chamber. The "cylinder" is tube No. 2, and the "piston" of the pump is the fluid in tube No. 2, which moves up and down as the rubber tube is rhythmically compressed by the magnet and its attached mechanism and released to expand by its own elasticity. The connecting channels (2 and 3) are between the valve chambers and the "cylinder." Channel 1 carries blood to and Channel 4 carries blood away from the chambers. The action of the valves and the direction of flow is easily seen, by reference to Figs. 5 and 6, to be that of an ordinary pump with the arrangement such as to propel the blood in the direction of the arrows.

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Tubes 1 and 3 are attached by short channels (12 and 13) to the valve chambers. All three tubes are furnished with pinchcocks. The pinchcock on Tube No. 2 is close to the plates by which the tubes are compressed and is kept closed during the operation of the pump. The pinchcocks on Tubes Nos. 1 and 3 are adjustable as to height. On No. 1 it is kept low except when the tube is being compressed as an aid in filling the chamber. Tube No. 3 serves to modify the impulse given to the blood. When the pinchcock is low the blood is propelled in short violent spurts; as it is raised higher and higher the rubber tube by its cushionlike elasticity more and more softens and prolongs the spurts, until a continuous pulsating flow may be obtained without reducing the total pressure.

The intermittent current for energizing the magnet is produced by interrupting a direct current by means of a mercury switch carried by a pendulum, or by any suitable mechanism, so adjusted that the time is equally divided between compression and release.

The reservoir is shaped as shown in order to reduce to a minimum the area on which blood corpuscles might stagnate and also to keep down the volume of blood necessary to operate the apparatus. The lower narrow part of the reservoir holds about 8 c.c.; since about 4 c.c. of blood is required to fill the pump chambers and all the channels, operation can be carried on with a minimum of about 5 c.c., although it is safer to use 8 to 10 cubic centimeters. (A rat donor weighing 500 grams furnishes sufficient blood for one experiment.) Around the reservoir and the return tubes is placed a larger cylinder fitted into a tapered groove for protection. It can serve as a reservoir in case larger quantities of fluid are required.

For oxygenating the blood the upper wide part of the reservoir is employed. When a small organ is being perfused it is sufficient to fill this part of the reservoir with small glass balls and to allow the returning blood to trickle over them, while at the same time oxygen released below rises between the balls to come into contact with the blood. However, when a large volume of blood is to be oxygenated, as in the perfusion of the stomach, pancreas, and part of the duodenum, a more efficient yet simple method had to be devised. This is shown in Fig. 1. It consists of a pair of windshieldlike wipers of rubber dam which smear over the inner surface of the wide part of the reservoir the returning blood which is directed by small pieces of stainless steel against the surface. The wipers are attached to a piece of glass tubing through which oxygen is conducted and released below the wipers. The glass tubing is fitted at its upper end with a brass sleeve which turns in a bushing in the glass cover of the outer cylinder. The sleeve carries a small gear which meshes with another gear, outside the cylinder, attached by a flexible cable to the reduction gear of a small motor. The wipers make about 20 complete rotations per minute and keep the blood spread in a thin film in direct contact with oxygen.

Blood is drawn by the pump from the reservoir through Channel 1 and is discharged through Channels 4 to 6 to the arterial spout and canula and thence into the artery of the organ. Having circulated through the vessels of the organ, it is discharged as dark venous blood through the venous canula to Channels 9 and 10 and on through the venous return tube to the top of the reservoir where

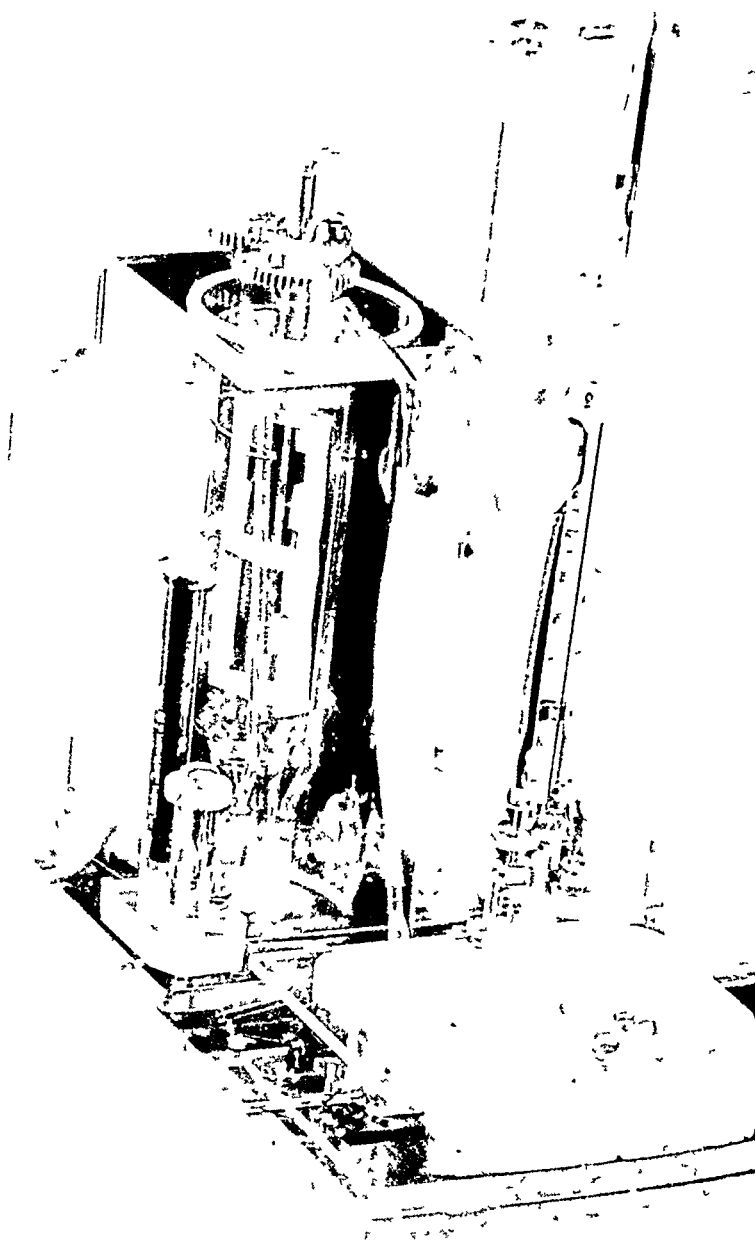


FIG 1.

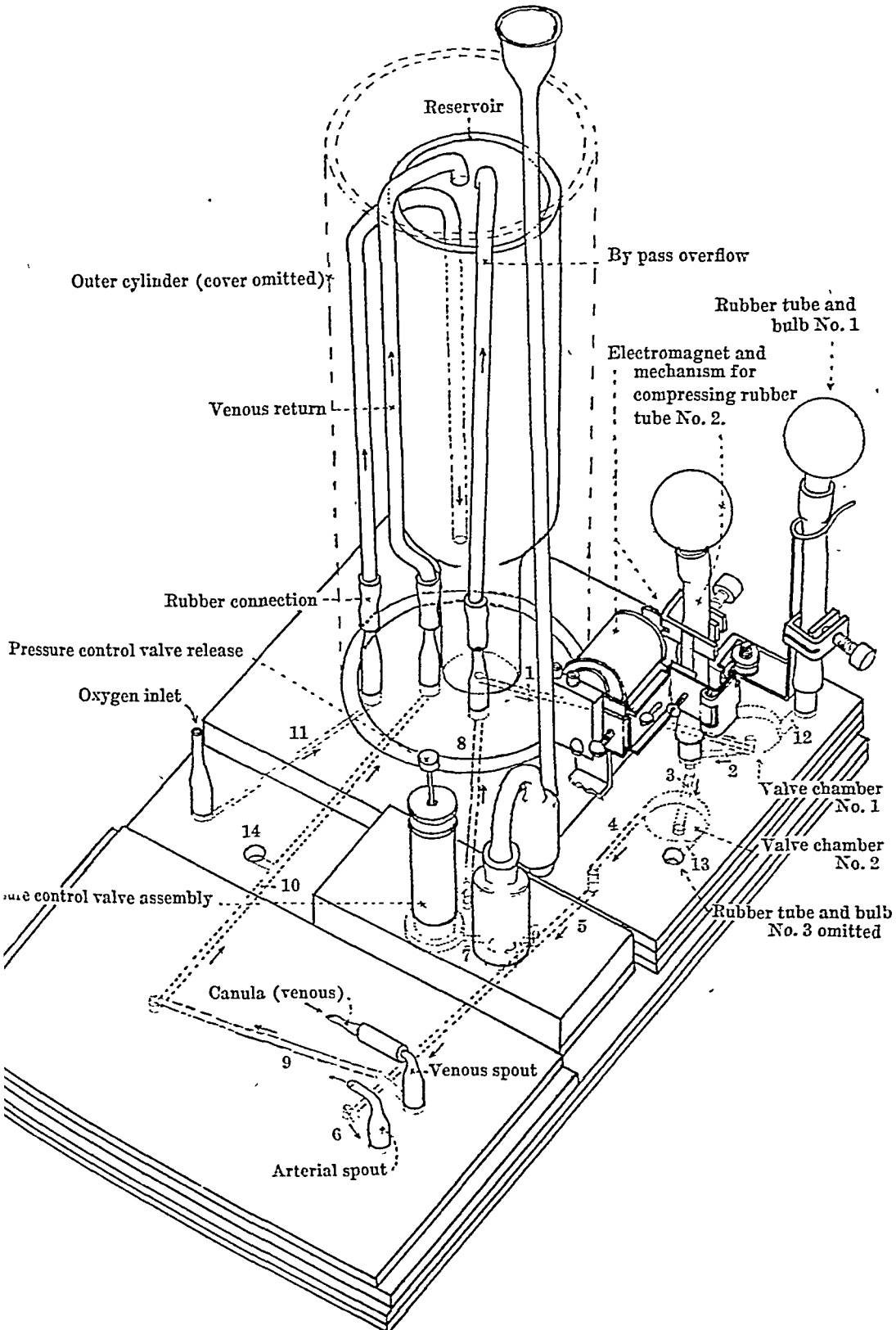
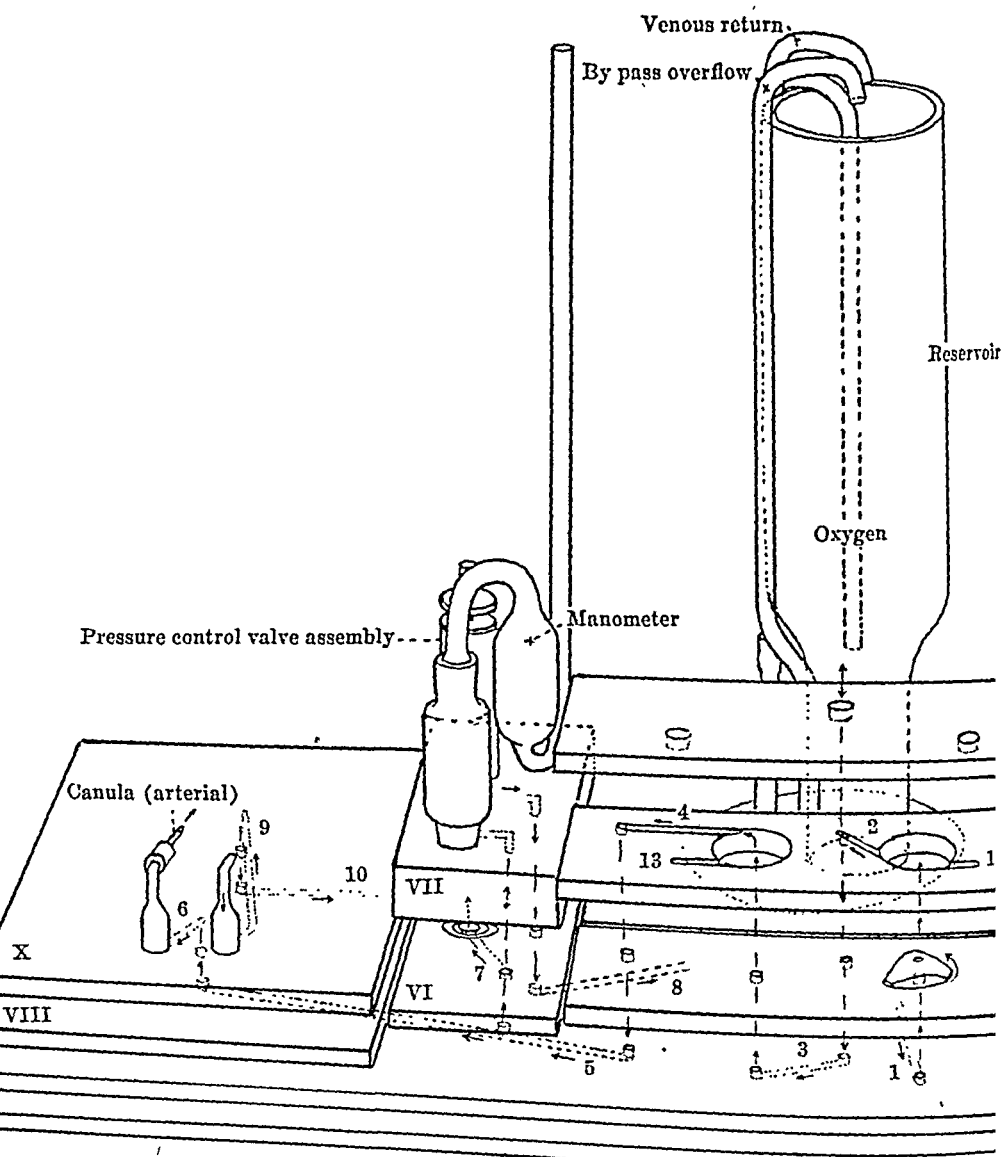


FIG. 2.

it spills over the glass balls or is spread over the inner surface. This circulation is all that would be necessary if the pump could be made to deliver just the exact amount at the desired pressure. But since the pump delivers blood in excess of the capacity of the organ, the excess must be bypassed, under control,



Valve No. 1 in place over hole in plat

Fig. 3.

through a side channel. This is accomplished by Channel 7 which passes from Channel 5 to open under a valve (Figs. 4 and 6) which is held down by an adjustable spring in the pressure control assembly. Blood which passes this valve is carried through Channel 8 to the bypass overflow tube also to the top of the

reservoir. It is arterial blood and helps greatly to keep the blood in the reservoir oxygenated. By means of this control valve pressure in channels between the pump and organ can be adjusted to the optimum desired, for the pump is capable of giving a pressure of over 200 mm. of mercury. The pressure is indicated by a

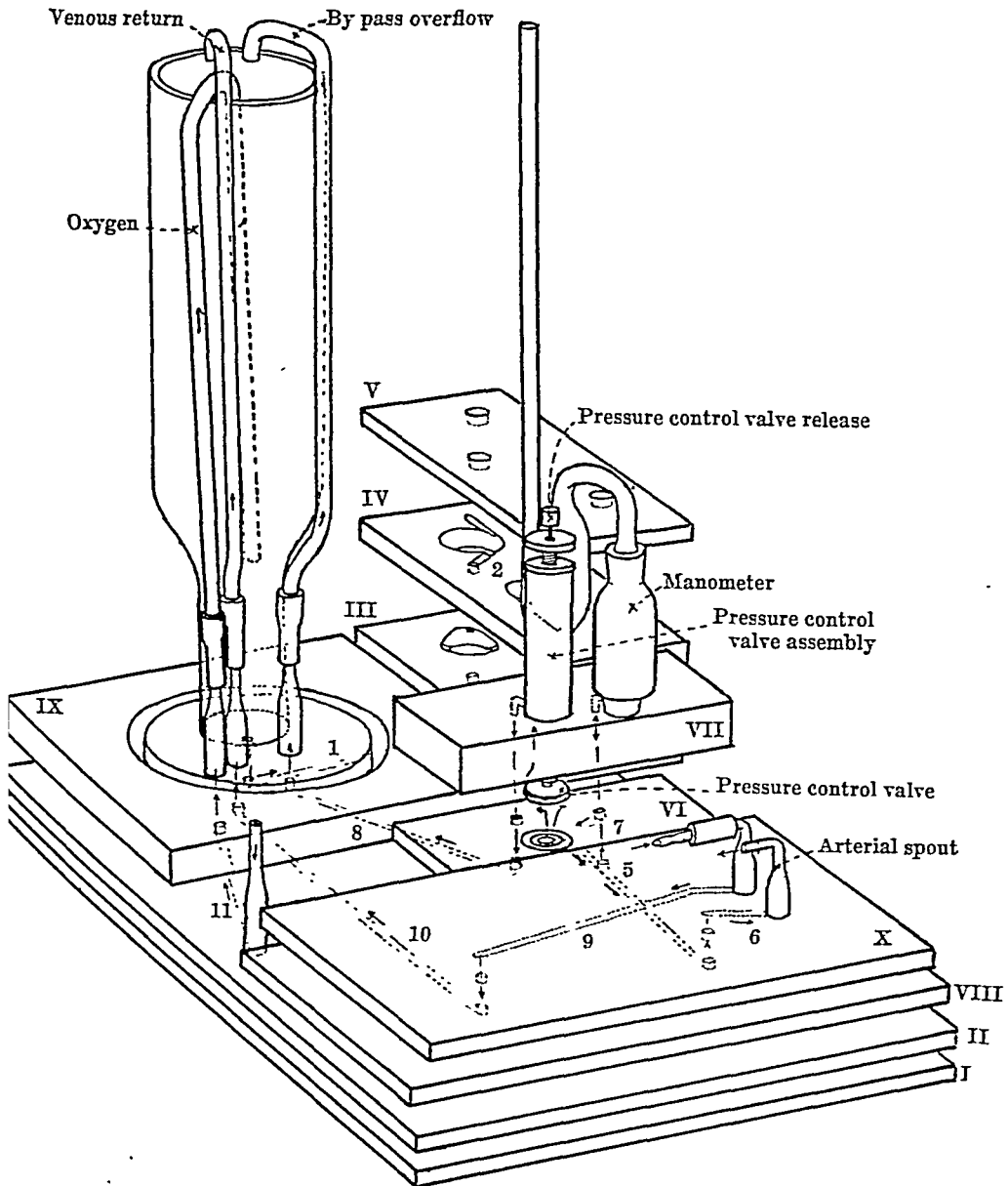


FIG. 4.

mercury manometer on another side channel between Channels 5 and 7. The arrangement of the manometer is sufficiently clear in Fig. 6, where it will be seen that there is an oil trap between the blood and mercury. It may be added that



the flask containing both blood and oil can be rotated in Plate VII, the lower portion thus serving as a stopcock when it is desired to isolate the manometer from the rest of the apparatus.

It has been found that in order to have a uniform and perfectly reliable action of the pressure control valve it is necessary to place a small disk of rubber dam between the valve and valve seat. Similar disks under the valves of the pump are advantageous.

For occasions when it is necessary to reduce the pressure quickly, a slender rod is provided attached to the valve, the raising of which at once releases the pressure in channels between the pump and specimen organ. By a simple mechanism shown in Fig. 1, this release is made automatic when the pressure becomes too high. A pair of fine enameled wires is inserted in the upper part of the manometer tube, down to the mercury level allowable. The wires are connected to an electromagnet. When the rising mercury column completes the circuit through the wires, the magnet jerks the release rod sufficiently to release the pressure.

For conveniently taking blood samples for analysis from the venous return Channel 10, at 14 (Figs. 2 and 5), is a short side channel and tapered orifice for a connecting tube or spout. The latter is connected by a short rubber tube to a three-way stockcock (Fig. 1) which, with a syringe arranged for blowing out the channels, makes it possible to take samples and to empty all channels from the outlet to Channel 10 so that each sample is uncontaminated by a preceding one.

At 15 (Fig. 5) is a channel from an outside tapered orifice, furnished with a spout, to an opening in the floor of the reservoir. To the spout a graduated cylinder is attached by a rubber tube (at the left in Fig. 1) through which the reservoir can be filled, and also emptied, with a measured quantity of blood.

Of equal importance with the rest of the apparatus are the means of supporting and attaching the organs to be perfused. This is accomplished by the specimen chamber together with Plate X on which it sets and its cover. The specimen chamber is made of plaster of Paris soaked in shellac and dried. The depressions in it are shaped (as in that for the stomach, duodenum, and pancreas, shown in Fig. 1) in such fashion as to support the organs in approximately their normal topographical positions, with provision to spread part of the pancreas over a shelf just under the cover where it can be observed under a microscope and to require the minimum amount of fluid to cover them. At appropriate locations are spouts for the blood canulae and outlets for the esophagus and duodenum. These are of stainless steel and are attached to Plate X. The outlets have connections to the outside so that fluid can be introduced into the stomach and collected from the intestine. The upper ends of the spouts appear at the right in Fig. 1. They are similar to those shown in Figs. 2 to 4 and have similar connections with the channels. In the plaster is embedded a coil of nichrome wire for heating, and two holes are provided for a thermometer and a thermostat. The size and form of the chamber and the location of the spouts differ with the organ under investigation.

In Fig. 1 may be seen a rubber and glass connection between the chamber and the upper end of the reservoir. This connection serves to return to circulation blood that accumulates in the chamber from small leaks, for it is practically impossible to prevent the escape of blood from minute tears or from seepage. The amount of this leakage varies greatly, and in no case is of importance, since it does not interfere with the supply of blood to the tissues.

The whole apparatus rests on a warm plate of approximately the correct temperature. The warm plate and the heating element and the thermostat in the chamber keep the latter at a very constant degree of heat.

The plates are sealed together with paraffin introduced when the plates are hot, with the result that all the channels, except those in the pump, are lined with a film of paraffin which keeps blood from contact with the glass, an item of some importance since most of the channels are in ordinary glass. Only certain parts are of Pyrex, namely, the pump, Plate IX into which the reservoir is set, and all parts made of tubing. In the ordinary course of sealing these Pyrex parts would not get the film of paraffin. The spouts and the reservoir are usually sealed in place with a mixture of beeswax and rosin.

The whole apparatus can be completely sterilized by appropriate methods. When it is to be thoroughly cleaned it is easily taken apart in hot water, washed with soap, and finally dried from alcohol with chamois skin before being assembled in a dustproof chamber. For work which does not require aseptic precautions it is sufficient to remove only the top plate of the pump (Plate V, Figs. 3, 4, and 6), manometer and pressure control valve and their plate (VII), the reservoir and spouts, and Plate X, along with the specimen chamber, to clean them and to rinse out the remaining channels, all of which can be done in a short time. These plates when reassembled are sealed with stopcock grease.

Since the construction of the apparatus (especially the specimen chamber, the spouts, and canulae) and the technique of isolating the organs have been developed together, it is appropriate to give a brief outline of the procedure of making the preparation. All of the dissecting must be done under a binocular microscope arranged to be focused by the feet of the operator. Minute electric cauteries, which can be turned on and off and the temperature controlled by the action of the right knee, are used almost exclusively for cutting mesenteries, vessels, and other tissues. During the isolating of the organs, which requires about one and one-fourth hours, it is important that the blood supply to organs, for example the stomach, pancreas, and that part of the duodenum to which the pancreas is attached, be maintained without interruption. The canulae used are short with bulbous tips and are attached to short pieces of rubber tubing (Figs. 2 to 4) which can be slipped over the spouts. Before being inserted into the blood vessels the free ends of the rubber connections are closed with minute stainless steel plugs, and the canulae and connections are filled with a solution of heparin. These canulae are introduced and tied into the portal vein before the latter reaches the pancreas and into the aorta just caudad to the left renal artery. Ligatures are placed in readiness around the portal vein where it enters

three diuretics employed. Ascorbic acid when given prior to the administration of mercurhydrin or simultaneously with it increased the average minimal lethal dose over 50 per cent. The lethal dose of mercupurin and of salyrgan with theophylline was not affected when combined with ascorbic acid.

Ascorbic acid previously has been noted to increase the diuretic effect of mercupurin. A study of ten persons in congestive failure who have received mercurhydrin with ascorbic acid intravenously has revealed an equal or increased diuresis up to 50 per cent.

These investigations indicate that mercurhydrin (2 c.c.) combined with ascorbic acid (500 mg.) is the least cardiotoxic and most potent intravenous diuretic preparation studied.

#### DISCUSSION

DR. EDWARD MASSIE, St. Louis, Mo.—I would like to ask whether vitamin C may be given without danger in the same syringe?

DR. JOHN W. SCOTT, Lexington, Ky.—I have found clinically that the action of the mercurial diuretics can be smoothed out by giving the patient one of the B complex lyophilized preparations dissolved in the mercurial product. This makes a clear solution which has been perfectly harmless in the 100 or more instances in which I have used it.

DR. CHAPMAN.—The workers in South America have used magnesium sulfate. While their report on results so far as percentages are concerned has not been given, there was some decrease in toxicity when these products were used in combination.

We have given vitamin C and the mercurial product in the same syringe with no bad effects.

Our experience has been the same as Dr. Scott's in the use of vitamin B complex together with the mercurial substance; it does help to smooth the use of the mercurial diuretic, clinically at least.

#### THE SERUM PROTEIN FRACTION RESPONSIBLE FOR THE THYMOL TURBIDITY TEST

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MADISON, WIS.

The thymol turbidity test of Maclagan (1944) has been shown to be of considerable diagnostic value in cases of liver disease. Maclagan stated in his paper that the test was probably due to an increase in serum gamma globulin. Recant and associates (1945), on the other hand, have shown that the test depended on the presence of a lipid-containing fraction of serum globulins rather than on the gamma globulins.

A systematic study in this laboratory of the serum protein fraction responsible for the thymol turbidity test indicates that a beta globulin is involved. A large number of fractions obtained from pooled normal human plasma were tested with the thymol reagent. High values were always associated with those fractions containing a high percentage of beta-1 globulin. Electrophoretic analysis of sera showing high thymol turbidity values invariably showed a high beta globulin level, whereas sera having normal beta globulin levels gave normal turbidity values. After mixing positive sera with thymol reagent and then dialyzing against the thymol reagent, it was possible to centrifuge off the precipitate from the supernatant and examine both in the electrophoresis ap-

paratus. The serum after treatment with thymol reagent, and free of its precipitate, differed from the untreated serum chiefly in a decrease of the beta globulin fraction to a normal level (Table I). Further, electrophoretic analysis of the precipitate after redissolving showed it to be chiefly a single protein with an electrophoretic mobility characteristic of normal beta globulins.

TABLE I. ELECTROPHORETIC ANALYSIS OF SERUM COMPONENTS BEFORE AND AFTER TREATMENT WITH THYMOL REAGENT

	ALBUMIN	PER CENT COMPOSITION			GAMMA
		ALPHA-1	ALPHA-2	BETA	
Before	47.7	4.5	9.1	19.1	19.5
After	53.3	5.5	9.1	13.0	19.0

While the beta globulin fraction of blood is known to be lipid-containing, the total lipid content of sera did not appear to be related to the thymol turbidity values. Thus, a serum from a case of lipid nephrosis with a cholesterol content of 1,400 mg. per 100 c.c. showed a negative test.

In a comparative study of cases of liver disease showing a discrepancy in the thymol turbidity and cephalin-cholesterol flocculation tests, the latter was invariably positive in cases of high gamma globulin levels and the former in cases of high beta globulin levels. In cases where both tests were positive both these fractions were elevated above normal levels. In general it was found that higher and more consistently positive thymol turbidity values were associated with acute inflammatory rather than chronic degenerative hepatic disorders.

#### DISCUSSION

DR. WILLIAM S. HOFFMAN, Chicago, Ill.—It would appear from Doctor Cohen's data that a positive thymol turbidity test reduces the beta globulin concentration of the serum to a level below normal. I wonder if this indicates that the positive thymol turbidity test is produced by the presence of a substance which, when thymol is added, precipitates the normal beta globulin.

#### BIOPSY OF LIVER IN PATIENTS WITH ACTIVE BRUCELLOSIS: DESCRIPTION OF HEPATIC LESIONS

FREDERICK W. HOFFBAUER, M.D., AND WESLEY W. SPINK, M.D.,  
MINNEAPOLIS, MINN.

A review of the literature on the alterations of the tissues in patients with brucellosis indicates that the changes are widespread involving, particularly, the reticulo-endothelial system. Practically all the reports have been based upon an examination of post-mortem material. Only on rare instances have biopsies of the liver been made in living subjects. The present report is part of a study on the pathogenesis of brucellosis and represents an endeavor to define the incidence and nature of the hepatic lesions in patients with active brucellosis.

Thus far, one patient with acute brucellosis and four with the chronic form of the disease have been studied. Four of the patients were proved to have brucellosis by isolation of *Brucella abortus* from the blood stream. The fifth individual had clinical and laboratory findings consistent with chronic brucellosis. In one subject a specimen of liver was obtained by means of peritoneoscopy;

in a second individual material was acquired incidental to an abdominal operation; and in the three remaining patients biopsies were made by an operative procedure under local anesthesia.

Abnormal histologic findings were apparent in all specimens. The liver of each of the five patients showed the presence of granulomatous lesions which have been described in brucellosis. The granuloma were usually found in the liver cords rather than in the portal spaces. These lesions were composed of epithelioid cells, lymphocytes, and plasma cells arranged in small round groups. In addition, variable increases in portal connective tissue and occasional necrotic liver cells were seen.

In an endeavor to correlate the hepatic injury with liver function, multiple liver function tests were carried out which included quantitative serum bilirubin, bromsulphthalein excretion, cephalin cholesterol, thymol turbidity, urine urobilinogen, and urine coproporphyrin. The degree of functional impairment in the five cases studied was slight contrasted with the extensive histologic changes. These observations indicate that hepatic changes may occur more frequently than is generally recognized in patients with active brucellosis.

#### STUDIES IN CONVALESCENCE FOLLOWING HERNIORRHAPHY: THE EFFECT OF DIET, SUPPLEMENTS, AND AMBULATION ON METABOLIC CHANGES AND ON THE PERFORMANCE OF PATIENTS AS MEASURED BY TESTS OF CARDIOVASCULAR EFFICIENCY AND MUSCULAR FITNESS

R. W. KEETON, M.D., W. H. COLE, M.D., N. O. CALLOWAY, M.D., N. GLICKMAN, M.S., H. H. MITCHELL, PH.D., J. DYNIEWICZ, PH.C., AND D. HOWES, B.S., CHICAGO AND URBANA, ILL.

An attempt was made to determine the extent and duration of convalescence from surgical trauma and to find means of shortening it. The progress of convalescence was measured by a study of certain metabolic factors and performance tests.

The liver was the organ primarily affected in ninety-seven healthy men submitted to herniorrhaphy. The decrement in liver function, as measured by twenty-four hour urobilinogen excretion, bromsulphthalein retention, and other tests, lasted about five days and returned slowly to normal. The decrement was considerably greater in patients who developed complications with infections in chest or wound. The decrement could be prevented by high protein and high caloric feedings, combined with ambulation, systematic exercises, and the administration of certain supplements.

The negative nitrogen balance usually observed postoperatively in patients of the control group on the usual hospital regime was prevented by the same measures which prevented the decrement in liver function. Ambulation alone caused a shift in nitrogen balance toward the positive side.

Attempts to use 17-ketosteroid excretion, creatine excretion, creatinine excretion, renal tests, and many additional liver-function tests were of little clinical value.

The cardiovascular efficiency was measured by the response of the patient on the tilt-table, stair-climbing test, step-up test, and an over-the-bed, push-up test. A single, numerical value has been developed for expressing the cardiovascular response to tilting.

Patients who were on the program of full ambulation exhibited less cardiovascular impairment than those on a program of modified ambulation or those on the control program. Patients who received adequate quantities of proteins and calories throughout the postoperative period (tube fed) also showed less impairment than the controls. This indicates that adequate proteins and calories exerted a favorable effect on circulatory efficiency.

The strength of hand grip, the endurance time of hand grip, and the response to selected psychomotor tests showed no significant decrements.

The expected temperature and pulse rate variations of patients exhibiting normal convalescence will be illustrated and described.

The totality of the results indicates that a herniorrhaphy is a slight operative load. It is a more useful procedure for studying metabolic changes than those revealed by performance tests. It is an excellent experimental procedure, since it is possible to secure adequate controls and the patients can all be brought to a normal state of health prior to operation.

#### DISCUSSION

DR. RICHARD M. JOHNSON, Long Beach, Calif.—I want to ask what you mean by high caloric intake. There seems to be some doubt if nitrogen balance can be maintained on less than 1,500 calories a day, so I would like to find out how many calories you give. I think that this is an important study, and in any study of this sort it is important to know how much activity the patient has and how much his muscles are used.

DR. M. A. BLANKENHORN, Cincinnati, Ohio.—I would like to ask the essayist if he has compared all wound infections with infections of the lung with atelectasis. I have in mind that a lung infection might result in an anoxia. This might be an important determining factor in the urobilin output. I would also like to ask if these were all urine urobilin determinations and whether any accounting was made of the stool urobilin.

DR. WARREN B. COOKSEY, Detroit, Mich.—Are there any preliminary reports on more prolonged or more chronic illnesses? I did not understand whether these patients were in the younger age group or whether they were an army group. Certainly Dr. Keeton and his co-workers deserve our commendation. I have the feeling that clinically an organized convalescence is of great value, but I know how difficult it is to get any support for a program of this sort. I can testify to this after seventeen years of attempting through subsidy and endowment to create a really good convalescent facility for Detroit, which is, I believe, a typical American city in this respect. We are barely able to keep a thirty-five bed endowed institution full of true convalescents. We are flooded with chronic and senile cases. We must pay more attention to the convalescent problem, and Dr. Keeton's work is an excellent start.

DR. CECIL J. WATSON, Minneapolis, Minn.—I judge that there is less urobilin excreted in patients who have been given spinal anesthesia. What type of inhalation anesthesia was used in the other patients?

DR. KEETON.—I am quite certain that the problem of calories is important. We regard the basal caloric requirement plus 20 per cent as a maintenance or just maintenance diet. As a matter of fact, these patients lost weight on that diet. When we talk about high caloric diets we mean basal plus 100 per cent. If the basal requirements are 1,700 calories and the

patient is fed 3,400, that should be more than sufficient to take care of the increased requirements associated with operation.

As to the age group of these patients, they were very largely young men who were furnished to us through the draft boards. Consequently, we believe that this constitutes a good control group for the study of operations because the subjects were relatively normal. I appreciate the suggestion made that when we start on most operations we are working on sick individuals and it becomes more difficult to study the patient's physiology prior to his operation.

We have not made any careful study of patients' response based on the types of infection. I simply gave you all the infections which arose in the course of this operation. We have recently agreed that in order to standardize further our procedures we would use penicillin as a prophylaxis for postoperative pulmonary infections.

The anesthetic which was used was nitrous oxide induction with ether. Pontocaine was used for the spinal anesthetic. No determinations were made of the urobilinogen in the stools.

## RELATION BETWEEN STRUCTURAL AND FUNCTIONAL ALTERATIONS OF THE LIVER

HANS POPPER, M.D. (BY INVITATION), MURRAY FRANKLIN, M.D.

(BY INVITATION), FREDERICK STEIGMANN, M.D., AND

DONALD D. KOZOLL, M.D., CHICAGO, ILL.

In eighty-five patients suffering from various hepatic diseases (infection and toxic hepatitis, cirrhosis, benign and malignant extrahepatic obstruction and other hepatomegalies), liver biopsies were performed by needle; they were repeated in twenty-seven instances, sometimes by surgical excision. Prior to each biopsy several liver function tests were performed. In general, the histologic diagnosis agreed with that derived from clinical and laboratory observations. Occasionally, the histologic differentiation of biliary hepatitis due to extrahepatic obstruction from infectious and particularly toxic hepatitis was difficult. Sometimes the clinical impression was corrected by the histologic findings.

To evaluate morphologically the liver function tests, the degree of each histologically demonstrable change was plotted against the result of each test and statistically evaluated. From the material examined so far, a close correlation appeared between diffuse damage of the epithelial liver cells (characterized by histologic criteria) and pathologic results in cephalin cholesterol flocculation, thymol turbidity, and bromsulfalein tests; between moderate relation to reduction of prothrombin time, increase of serum bilirubin, and reversal of A/ ratio; and between slight relation to increase in sedimentation rate, urinary urobilinogen, N. P. N., and decrease in cholesterol ester ratio and serum vitamin A. No quantitative relation appeared to serum alkaline phosphatase, total cholesterol, fecal urobilinogen, and direct-indirect bilirubin ratio.

In contrast, focal parenchymal necrosis bore a slight relation only to cephalin flocculation, thymol turbidity, and serum vitamin A, indicating that focal damage, even if more severe than diffuse liver cell damage, may escape recognition by function tests. Regeneration of the liver cells showed a significant relationship to thymol turbidity and a questionable one to cephalin flocculation and serum vitamin A. Regeneration was more often found when dro

in cholesterol esters and A/G reversal were moderate than if advanced. Reconstruction of the lobular pattern appeared related to cephalin flocculation, less to thymol turbidity. With marked inflammatory activity in the periportal fields, the sedimentation rate was uniformly elevated; the former bore some relation to cephalin flocculation, increased serum bilirubin, and reduced prothrombin time. Its relation to thymol turbidity and urinary urobilinogen was equivocal. Increased activity of Kupffer cells coincided only somewhat with A/G reversal, reduced cholesterol esters, increased serum bilirubin, and elevated thymol turbidity.

Although obviously marked overlapping occurs between histologic changes in the individual patient, it is nevertheless hoped that this morphologic approach may help in the diagnosis of hepatic disease by establishing relations between results of function tests and the different pathologic phenomena within the liver.

#### DISCUSSION

DR. CECIL J. WATSON, Minneapolis, Minn.—Dr. Hoffbauer and I often have been impressed with the striking lack of correlation between histologic evidence of disease and changes as reflected by some of the functional tests. This was first borne in upon us several years ago in studying liver damage due to sulfanilamide. In many of these cases we saw outspoken jaundice with other evidence of liver dysfunction but with surprisingly little histologic evidence of impairment. Conversely, we often have seen instances of cirrhosis of the liver in which there was but little evidence of functional impairment in spite of marked histologic derangement.

I should like to emphasize that group of cases of late or chronic hepatitis with jaundice which is perhaps best designated as cholangiolitic hepatitis, in which liver cell function has returned to normal and the appearance of the liver cells as studied by means of biopsy is found to be normal. One often sees little in the biopsies from this sort of case, although the condition is inflammatory. Because of marked evidence of regurgitation of bile in the blood, one can only conclude that there is a functional injury of the cholangiolar epithelium.

I notice that Dr. Popper has used a simple Ehrlich test and I presume that the test was carried out on single samples or short period collections of urine. I think the upper limit of normal which he gives as 3 units is too high. We studied sixty normal students and in that group found that 95 per cent had less than 1 unit in two-hour samples from 2 to 4 in the afternoon; the rest ranged up to 1.5 units. We are inclined to believe that 1.5 units represent the upper limit of normal, although there may be borderline cases up to 2.0 units. We have regarded anything over 2 units as distinctly abnormal.

I should like to emphasize that this procedure has limited value. It has a good deal of value because of its simplicity and the ease with which it can be applied to a large number of individuals. On the other hand, we have seen individuals with liver disease who had a normal value over a short period of time, and yet in the same twenty-four hour period the urobilinogen might show a considerable degree of elevation.

DR. FREDERICK W. HOFFBAUER, Minneapolis, Minn.—I should like to compliment Dr. Popper and his associates on this study. I would like also to ask about the distinction between extrahepatic biliary obstruction and hepatitis on the basis of biopsy. We have thought that if the biopsy could be secured early in the course of the jaundice the distinction would be possible, but when the biopsy is made after the patient has been jaundiced for three or four weeks, the pathologist will have considerable difficulty distinguishing between the changes that occur in extrahepatic obstruction and those that occur in hepatitis.

I should like also to ask about the degree of depression of cholesterol esters in patients with biopsy evidence of fatty livers. I am sure the surgeons would welcome some evidence



that would denote or at least lead them to suspect fatty infiltration of the liver. How extensive were the changes in the cholesterol ester fractions?

DR. LEON SCHIFF, Cincinnati, Ohio.—I would like to ask Dr. Popper if he has noticed any specific changes in the livers of those patients with hepatitis in whom the jaundice appears to be completely obstructive in character. These cases present laboratory changes that cause them to be confused with cases of obstructive jaundice due to stone or tumor. Is there any specific change in the liver biopsy specimens in this particular group of hepatitis often called cholangiolitic form of hepatitis?

DR. POPPER.—First, I want to stress once more that in a certain number of patients examined, discrepancies between anatomical and functional changes were found. For instance, a certain percentage of patients who revealed liver function impairment did not show liver cell damage in the biopsy specimen and vice versa. What we are attempting is a statistical approach comprising all cases.

We can confirm the observations of Dr. Watson as to the occurrence of the morphologic picture of cholangiolitic cirrhosis without significant disturbance of many of the liver functions. We have five cases of this type on record.

As to the question of histologic differentiation between the various types of hepatitis, we found the morphologic differentiation between infectious (viral) hepatitis from toxic hepatitis due to a variety of causes not too difficult to make. We believe we have histologic criteria as well for the fatal as for the nonfatal form of these diseases. On the other hand, the differentiation between toxic hepatitis from biliary hepatitis due to extrahepatic obstruction is sometimes difficult. We have made most of our mistakes in the interpretation of liver biopsies along this line. We are trying, at present, to develop histologic criteria by obtaining additional experience on those cases. One reliable evidence for a biliary hepatitis due to extrahepatic obstruction is the so-called bile infarct which represents necrotic areas impregnated with bile. It does not occur in other groups. We are at a loss to explain the morphologic cause of intrahepatic biliary obstruction and we accept the explanation of Watson and Hoffbauer, namely, that of a morphologically unrecognizable reflux through the bile ducts.

We are encountering difficulties in the chemical determination of cholesterol esters in using the colorimetric method. In our material fatty changes were often accompanied by a decrease in the cholesterol ester percentage. On the other hand, we occasionally saw severe hepatitis with little decrease of the ester percentage.

## HEPATITIS WITHOUT JAUNDICE IN INFECTIOUS MONONUCLEOSIS

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CHICAGO, ILL.

From reports of punch biopsies, the jaundice occasionally associated with infectious mononucleosis has been shown to be due to parenchymatous changes in the liver not unlike those seen in infectious hepatitis. Similar changes in the liver have been reported in a nonjaundiced case of infectious mononucleosis. Cohn and Lidman (1946) found evidence of impaired liver function in fifteen consecutive cases of infectious mononucleosis without jaundice.

We have performed serial liver function tests in nineteen consecutive cases of infectious mononucleosis without jaundice (icterus indices of 10 units or less). Some degree of hepatic dysfunction was present in all cases as shown by the cephalin-cholesterol flocculation and sulfobromophthalein excretion tests. The maximum dye retention was 36 per cent in one hour. An abnormal albumin-globulin ratio was present in two out of ten cases in which it was determined.

There was a correlation between the severity of symptoms and the degree of liver damage. The usual duration of hepatic dysfunction was three to six weeks, but in three cases it persisted for three to four and one-half months. The liver was palpable and/or tender in eight of the nineteen cases.

The treatment consisted of rest, a high protein, high carbohydrate diet, and vitamin supplements. We have no evidence that the course of the disease was influenced by the diet. Patients who resumed moderate activity before the liver function returned to normal had no relapse of liver disease as indicated by liver function tests or by an increase in size and tenderness of the liver.

#### DISCUSSION

DR. A. E. FELLER, Cleveland, Ohio.—How was the diagnosis established in these cases?

DR. DeMARSH.—We performed serial white blood cell and differential counts in all of these patients. We made the diagnosis clinically first and confirmed it by heterophil agglutination tests. We did not do the absorption test. The clinical picture in each case was very typical and could not be confused with anything else.

#### THE EXPERIMENTAL PRODUCTION OF JAUNDICE OF THE DIRECT-REACTING TYPE BY THE INJECTION OF A PREPARATION OF DIRECT-REACTING BILIRUBIN

F. E. SNAPP, M.D. (BY INVITATION), M. GUTMANN, M.S. (BY INVITATION),  
T. W. LI, PH.D. (BY INVITATION), AND A. C. IVY, PH.D., CHICAGO, ILL.

By making an alkaline aqueous solution of crystalline bilirubin and mixing it with pooled canine plasma, and then injecting this alkaline bilirubin-plasma mixture into dogs, a direct-reacting type of jaundice, as occurs in obstructive jaundice, can be produced.

The amount of plasma and source of bilirubin is important; the best mixture is made as follows: 300 mg. of crystalline gallstone bilirubin is dissolved in 45 c.c. of N/10 NaOH, and the resulting solution is added to 300 c.c. of pooled reconstituted canine plasma. When this is given slowly intravenously, no reactions or changes in body temperature have been observed in twelve dogs. For some unknown reason about twice as much bile bilirubin as gallstone bilirubin has to be used to give an equivalent blood retention of the bilirubin (thirty-four tests).

By giving an injection of 200 to 300 mg. of bilirubin plasma every third or fourth day, or smaller doses every day, the jaundice was maintained in three dogs for four weeks. The jaundice cleared in five to seven days after ceasing the administration.

When the jaundice was maintained by giving 50 mg. of bilirubin in 50 c.c. of plasma daily, the only change detected was a delay in rose bengal clearance. With larger doses, the rose bengal clearance was decidedly reduced in three dogs, the alkaline phosphatase was slightly increased in one, the sedimentation rate of erythrocytes was markedly increased in two and slightly in the other, and in four weeks a slight anemia occurred. The animals ate well and did not lose weight.

It is suggested that a method is now available for producing a direct-reacting type of jaundice in patients with arthritis by substituting human for canine plasma in the previously mentioned specifications with the idea of ascertaining whether a remission of the disease may be induced.

#### DISCUSSION

DR. CECIL J. WATSON, Minneapolis, Minn.—If this method were to be applied in a large series of cases it would be interesting to compare the two forms of jaundice which one might expect, that is, that which is produced immediately due to the injection of bilirubin and that which will ensue in some instances about three months later due to transmission of the virus of homologous serum hepatitis.

#### THE EFFECT OF METHIONINE UPON THE URINARY NITROGEN IN HUMAN BEINGS AT NORMAL AND LOW LEVELS OF PROTEIN INTAKE\*

RICHARD M. JOHNSON, M.D., H. J. DEUEL, JR., PH.D. (BY INVITATION), MARGARET G. MOREHOUSE, PH.D. (BY INVITATION), AND JOHN W. MEHL, PH.D. (BY INVITATION), LONG BEACH, CALIF.

Methionine offers considerable protection to the livers of experimental animals against hepatotoxic agents and is thought to be of some therapeutic value in the treatment of certain types of liver disease in man. Methionine has been observed to exert a protein-sparing action in the dog, rat, and chick. This study was undertaken to determine the protein-sparing action of methionine in man.

Eight volunteers were placed upon diets containing 2,000 calories and 11.9 and 2.3 Gm. of nitrogen with and without 3 Gm. d-1 methionine for one or more periods of ten days each.

All subjects were constantly observed by four nurses and an attendant who kept daily records of temperature, pulse, respiration, and physical activity and weekly records of body weight, blood pressure, plasma protein concentration, hemoglobin, and red blood cell counts. The daily urinary excretion of nitrogen, creatinine, and sulfate was determined and stools were weighed. The diets were analyzed for their nitrogen content.

Methionine did not significantly alter the urinary nitrogen or creatinine excretion in any subject under these conditions. About 80 per cent of the methionine given were accounted for by increased urinary sulfate excretion and 10 per cent were excreted as methionine in the urine.

Four volunteers from the Department of Biochemistry were given 600 calories or 1,200 calories containing 0.94 Gm. nitrogen. In this group methionine failed to exert a protein-sparing action. Ninety-six per cent of the methionine taken was accounted for by increased sulfate excretion.

*Conclusions.*—Methionine did not exert a protein-sparing action at 2,000 caloric intake level containing 11.9 and 2.3 Gm. of nitrogen.

Methionine did not exert a protein-sparing action at a 600 or 1,200 caloric level containing 0.94 Gm. nitrogen.

The methionine was metabolized.

\*This work was done under a contract between the University of Southern California and the Research and Development Branch, Military Planning Division of the Office of the Quartermaster General, United States Army.

A species difference for minimum requirements of methionine is suggested. The daily urinary nitrogen excretion was lowest at the 2,000 caloric intake level and greatest at the 600 caloric intake level.

## ULCER OF THE LEG DUE TO ARTERIOLOSCLEROSIS AND ISCHEMIA OCCURRING IN THE PRESENCE OF HYPERTENSIVE DISEASE (HYPERTENSIVE-ISCHEMIC ULCERS)

### A PRELIMINARY REPORT

EDGAR A. HINES, JR., M.D., AND EUGENE M. FARBER, M.D. (BY INVITATION),  
ROCHESTER, MINN.

A small group of patients has been observed who had painful ulcers of the legs which were difficult to heal and which did not fit into any of the usual clinical classifications of ulcers of the leg. The lesions did not have the appearance of typical stasis ulcers but were ischemic and similar to the ulcers which result from spastic or occlusive involvement of the smaller arteries such as occurs in chronic pernio, livedo reticularis, or senile changes in the skin. However, in this particular group of patients none had any of the commonly recognized conditions producing ischemic or stasis ulcers, and no dermatitis was present. The usual diagnosis was ischemic ulcer of undetermined cause.

After observation of several patients it was noted that, aside from the fact that they were all women with the same type of ulcer of the legs, they had one condition in common, namely, hypertensive disease of long duration and considerable sclerosis of the retinal arterioles of the chronic hypertensive type. It was considered that changes similar to those in the retinal arterioles could also occur in the small arteries of the skin and subcutaneous tissues and could give rise to small areas of infarction of the skin. As the result of trauma or for some unknown reason, the skin might break down and such an ischemic ulcer would form.

The first case in which we considered that the ulcer on the leg might be related to vascular changes in the skin due to long-standing hypertensive disease was observed in 1941. Up to the present time we have observed seven patients and have obtained from the file of the clinic records of four patients who had the typical ulcers and hypertensive disease of long duration and no other vascular or cutaneous disease which could account satisfactorily for the lesions. We are presenting this group of eleven cases as a possible new syndrome, with the hope that additional observation and study will further clarify the cause and treatment of these lesions.

Histopathologic study of specimens from the ulcers and of the adjacent skin in five cases revealed organic changes in the arterioles. The most common changes were an increase in the thickness of the arteriolar wall and a decrease in the diameter of the lumen. An increase in the number and size of the nuclei and hyaline degeneration in the media, intimal proliferation, and periarteritis were also noted. The changes paralleled those reported by Keith, Wagener, and Barker from their studies of the pectoralis major muscles in cases of essential hypertension. There were no significant pathologic changes in the sections from the tissue removed from the ulcers except with reference to the arterioles.

Tentative criteria for the diagnosis of hypertensive-ischemic ulcer of the leg should include: (1) hypertension, (2) an ischemic appearing ulcer, (3) indolence of ulcer, (4) moderate to severe pain, (5) poor response to conventional treatment, and (6) typical changes of the blood vessels in and near the lesion as indicated by biopsy. The following should be ruled out before the diagnosis is made: (1) significant chronic venous insufficiency, (2) occlusive arterial disease, (3) syphilis, (4) blood dyscrasia, (5) cutaneous sensitivity to drugs, frostbite, seasonal variations affecting the occurrence of the lesions, or serious local injury, and (6) other diseases on pathologic study of the lesion.

#### DISCUSSION

DR. ROY W. SCOTT, Cleveland, Ohio.—Will you tell us what therapeutic management you have found of most value in these cases?

DR. A. A. HOLBROOK, MILWAUKEE, WIS.—Several of the photographs of leg lesions looked like cutaneous diphtheria. I wonder if any culture studies were made.

DR. HINES.—In regard to treatment, we have considered these patients from the standpoint of an occlusive arterial disease. Treatment has included such vasodilators as heat or diathermy applied to other parts of the body than the lower extremities, typhoid vaccine administered intravenously to produce fever in the younger patients, and occasionally whisky given orally. I believe this approach has been helpful. Of the various local applications tried, and they have been numerous, it would seem that powdered blood cells worked the best. The ulcers were slow to heal but they did heal eventually.

Cutaneous diphtheria was considered in several cases. Cultures were made several times in each case and we found no special organisms in these cultures. I do not believe the condition can be considered as cutaneous diphtheria.

#### AORTIC STENOSIS: AN ANALYSIS OF 106 PROVED CASES

CARL KUMPE, M.D. (BY INVITATION), AND WILLIAM B. BEAN, M.D.  
CINCINNATI, OHIO

Recently two instances of advanced aortic stenosis with bizarre clinical findings were studied during a single week on the wards of the Cincinnati General Hospital. Because the diagnosis was missed we have undertaken a review of all cases of aortic stenosis without significant lesions of other valves which have come to autopsy in the Department of Pathology in the last twenty-six years. We found 106 cases from a total of 15,806 records. A survey of medical reports and textbook discussions of aortic stenosis revealed great emphasis upon the classical signs of basal systolic murmur transmitted upward, and thrill, but little or no consideration of the atypical case which in our experience has actually predominated. Correct diagnosis, unless established in a former admission, was rarely made in the presence of acute cardiac failure of either forward or backward variety because of the absence of classical signs. The most common mistakes in diagnosis were primary disease of the central nervous system (that is, cerebral vascular accident, epilepsy), acute myocardial infarction, and pulmonary embolism. Even when the diagnosis of aortic stenosis was correct, mitral stenosis was diagnosed frequently but not found post mortem.

The history, mode of onset, electrocardiogram, x-ray, clinical and laboratory data, course of the disease, and manner of death will be correlated with the

cardiac and other findings at autopsy. Although syncopal attacks and even transient hemiparesis were fairly frequent, dizziness and petit mal-like seizures were very common as were anginal pain and paroxysms of dyspnea. A terminal state of shock with symptoms referable to a variety of organs was encountered often. Advanced degrees of aortic stenosis were found to be compatible with a relatively long life, especially if the patient lived within the narrow margins of his cardiac reserve. With these points in mind recent experience has shown that the diagnosis of aortic stenosis can be made without the classical signs if the possibility is kept in mind. In fact the local trend now is in danger of swinging too far.

#### DISCUSSION

DR. ROY W. SCOTT, Cleveland, Ohio.—The difficulties in making a definite diagnosis in patients with congestive heart failure are well established. We all have missed these patients and have been surprised to find very marked aortic stenosis at the post-mortem table. I hope that Dr. Kumpe will comment on the question of whether calcific aortic stenosis is a distinct entity or whether it is always due, as some believe, to a preceding inflammatory process involving the aortic leaflets.

DR. KUMPE.—In reference to the question about calcific aortic stenosis we do not have any answer. We did not go into the pathology. Dr. Bean agreed that it was a good topic to avoid.

In reference to the question about pure congenital aortic stenosis, no case was considered to be congenital in this series.

As to the electrocardiographic changes, forty-four showed left axis deviation, seven no axis deviation, and one a transient early right axis deviation which later returned to no axis; there were twelve auricular fibrillations, and there was a P-R interval in twenty-one cases over 0.2 second.

#### AN ANALYSIS OF IMMEDIATE MORTALITY IN 572 CASES OF RECENT MYOCARDIAL INFARCTION

L. N. KATZ, M.D., AND S. S. MINTZ, M.D. (BY INVITATION), CHICAGO, ILL.

A series of 572 cases seen at the Michael Reese Hospital between 1940 and 1945 inclusive have been investigated to determine some of the factors involved in causing immediate mortality (during the hospital stay). Hypertension appears to have no influence on the immediate prognosis of acute myocardial infarction. Diabetes mellitus definitely adds to the gravity of the prognosis, especially in women. It increases the frequency of death by precipitating uncontrolled diabetes and ketosis. Angina pectoris preceding acute infarction has no significant effect on the immediate prognosis. However, the persistence of anginal pain after the attack makes the immediate prognosis graver. Pulse pressures of 25 mm. Hg or less are of great prognostic significance. A systolic blood pressure drop to 90 mm. Hg for several days is a poor prognostic sign. The presence of shock has a similar implication. In the hypertensive group a drop of systolic pressure to 100 mm. Hg for three to five days makes the immediate prognosis poor. The immediate mortality is greater in patients having an infarct involving the septum or one showing atypical or combined patterns. There is no significant difference in the mortality rate of anterior and posterior wall infarcts. In all types the mortality rate is higher in women than in men. Cardiac arrhythmias make the immediate prognosis grave especially when

quinidine and/or digitalis are exhibited. The most serious types of arrhythmias are frequent ventricular premature systoles and intraventricular block. The presence of congestive heart failure is a grave prognostic sign. There is an added hazard in the use of digitalis in such patients, the immediate mortality being higher in those patients who received digitalis. Thromboembolic phenomena occurring during recent myocardial infarction have a grave prognostic significance.

#### DISCUSSION

DR. ROY W. SCOTT, Cleveland, Ohio.—Do you have any data on the relation between the duration of shock after the initial attack and the ultimate prognosis? It has seemed to me that the longer the patient remained in shock the surer he was to die.

DR. CECIL STRIKER, Cincinnati, Ohio.—I would like to ask if Dr. Katz has any opinion on the use of anticoagulants as a factor against these mortality statistics.

DR. M. J. SHAPIRO, Minneapolis, Minn.—I should like to ask if Dr. Katz concludes that one should not use quinidine at all in the treatment of coronary thrombosis.

DR. MOSES BARRON, Minneapolis, Minn.—I would like to know how many of the patients showed embolic phenomena. I also would like to ask about the use of digitalis in coronary thrombosis, since Dr. Katz spoke of the dangers of its use. It is true that we have to be careful in the use of digitalis in these cases; however, when there is congestive heart failure digitalis should be used but in a smaller dose. Would this method also contribute to the death in such cases?

DR. HENRY T. RICKETTS, Chicago, Ill.—I should like to ask what evidence Dr. Katz has that the administration of insulin, short of producing hypoglycemia, is harmful to the patient with diabetes with myocardial infarction. If he believes that patients with infarction are benefited by hyperglycemia, is it his custom to administer glucose routinely?

DR. JOHNSON MCGUIRE, Cincinnati, Ohio.—How late in the course of convalescence did embolic phenomena occur?

DR. HAROLD C. LUETH, Omaha, Neb.—I would like to ask Dr. Katz if he made a study of the degree of cyanosis and its relationship to mortality in myocardial infarction.

DR. WARREN B. COOKSEY, Detroit, Mich.—I feel that there is indeed an occasional direct relationship between strenuous exercise and coronary thrombosis. Dr. Katz does not state the time element involved in his study. Surgeons know that several days may elapse before signs of thrombus develop after a blood vessel trauma, and this should be borne in mind in relation to coronary thrombosis. A few years ago a study of 100 patients under my care seemed to show that seventeen of them were sedentary individuals who had engaged in strenuous physical exertion within twenty-four to forty-eight hours of their signs of complete occlusion.

DR. ROY W. SCOTT, Cleveland, Ohio.—What about post-mortem evidence of hemorrhage?

DR. CARL F. SHAFFER, Houston, Texas.—What are your criteria for diagnosis?

DR. KATZ.—We have no adequate data on the duration of shock and the ultimate prognosis. I would consider, however, that the prognosis would be worse the longer the duration of shock.

As to anticoagulants, I think that the cooperative study which Dr. Wright has set up may settle this when 1,000 cases are carefully studied. My own feeling is that a patient who has had a thromboembolic phenomenon, after the original infarct, should receive anticoagulants. I might be persuaded to give it in all instances of recent infarction.

We use quinidine when there is an indication for it. Multiple premature systoles or paroxysmal tachycardia is one indication. However, quinidine is a dangerous drug and should be given cautiously in acute infarction.

Of the 572 cases in our series 107 patients died (21.8 per cent) and fifty-two had thromboembolic phenomena; of these twenty-nine died (55.8 per cent). Thromboembolic phenomena occurred in 10 per cent of all patients. Thromboembolic phenomena occurred in 27.1 per cent of the patients who died.

Of course we give digitalis. If there is an indication give it, give it slowly and carefully. We are not sure what the cause of death is in these digitalized patients; nevertheless, there is sufficient hazard connected with its use to avoid giving it to every patient with an infarct, unless the patient shows progressive congestive heart failure.

As regards insulin and glucose, I made the statement about the danger of giving insulin to patients with angina because such patients then do not do so well. In acute emergencies insulin is covered with glucose.

I have no specific data on when emboli occur during convalescence, but I would say it is within two weeks after the infarction.

We did not study virus infection in the lungs.

About physical exercise, I point to the work of Blumgart that infarction was found to ensue after unduly severe exercise in Army personnel. In our series one patient had a similar story. We admit that when we take a history there are omissions, that patients may not remember all the facts, and that the questions may prejudice the patient.

We have made no analysis of subintimal coronary hemorrhage in this series.

The criteria of location of a lateral wall infarction in the electrocardiogram is published in my book on electrocardiography.

The Michael Reese Hospital patient population is about equally divided between the two sexes, and the male dominance in infarction is therefore not dependent upon an unequal sex hospital population.

## PRESENT STATUS OF THE THERAPY OF EXPERIMENTAL RENAL HYPERTENSION\*

G. E. WAKERLIN, M.D., OLIVER KAMM, PH.D. (BY INVITATION), WAYNE DONALDSON, M.S. (BY INVITATION), W. G. MOSS, M.S. (BY INVITATION), HIROAKI MINATOYA, M.S. (BY INVITATION), TED LEFCO, B.S. (BY INVITATION), AND JOHN MARSHALL, B.A. (BY INVITATION),  
CHICAGO, ILL., AND DETROIT, MICH.

The Council has asked me to present a brief review of the recent research of our group at the University of Illinois with reference to the present status of the therapy of experimental hypertension produced by constriction of the renal arteries, and as more or less summarized in three abstracts in this year's Proceedings of the Central Society for Clinical Research.

The three therapeutic approaches which have received most attention in recent years have involved the use of naphthoquinones, marine and fish liver oils, and renal extracts. Several groups of investigators have reported the successful therapy of renal hypertensive rats and dogs with various quinones, especially the vitamin K naphthoquinones. There is at least one recent favorable report in the literature on the use of water-soluble vitamin K in essential hypertension. However, we have found no antihypertensive effect in renal hypertensive dogs from either fat-soluble or water-soluble vitamin K. This, of course, does not preclude effectiveness in human essential hypertension, since the question of a common partial pathogenesis of experimental renal hypertension and essential hypertension is still unsettled.

\*Aided by grants from the John and Mary R. Markle Foundation and the Graduate School Research Fund of the University of Illinois.



Following earlier clinical reports of the value of vitamin A concentrate obtained from fish liver oils in essential hypertension, we tested a vitamin A concentrate as well as a number of other vitamins in the treatment of experimental renal hypertension. None of these was effective except the vitamin A concentrate. Since then our group has tested three other lots of presumably the same vitamin A concentrate and found only one to be slightly antihypertensive. Of two other vitamin A concentrates tested, one showed slight antihypertensive activity. Eleven marine and fish liver oil fractions have proved to have no antihypertensive effect. Another research group has likewise found fish liver and marine oils inconstantly to contain variable amounts of an orally effective antihypertensive principle. This principle is definitely not vitamin A. Assay of its value in human hypertension should await further work on the separation, purification, and, if possible, identification of the principle and its production in adequate quantities.

A number of workers have reported the presence of an antihypertensive principle in kidney, but to date no one has produced a consistently effective, reasonably purified preparation properly suited for decisive trial in essential hypertension or human renal hypertension. Our research group has found a crude renal extract to be highly effective in the treatment of renal hypertensive dogs. At first we suggested that the antihypertensive effect was due to the *in vivo* neutralization of renin by antirenin produced as a result of the injections of renin contained in the crude extract, but more recent work makes the antirenin hypothesis very doubtful. Since we have obtained evidence that the antihypertensive activity may be in the nonrenin portion of the crude extract, we have studied about thirty fractions of the crude renal extract, some containing little or no renin. Certain of these fractions have proved to be inconstantly active, but we have not as yet been able to produce a consistently active purified preparation. On the basis of our work to date, we have demonstrated the following properties for the antihypertensive renal principle: (1) water-soluble (at least between pH 4.0 to 8.0); (2) retains at least partial potency for one year at pH 4.0 and 4° C.; (3) fairly resistant to slow freezing; (4) partially stable at 70° C. for twenty minutes; (5) poorly resistant to 40° C. for four hours under vacuum at pH 4.0; (6) probably nondialyzable; (7) partially precipitated by  $\frac{1}{12}$  saturation with  $(\text{NH}_4)_2\text{SO}_4$ ; (8) possibly a protein somewhat smaller in size than the pseudoglobulin renin.

We have likewise been able to substantiate our earlier observations that certain renal extracts given prophylactically will prevent the development of experimental renal hypertension in dogs. The technique of renal artery constriction which we have used gives a somewhat higher percentage of hypertension success than that reported by other research groups or obtained by us with slight modifications of the technique.

Recently we have been able to remove renin from the kidney biologically and are in the process of testing nonrenin kidney extract for the antihypertensive renal principle therapeutically and prophylactically. More work is obviously necessary before a sufficiently pure and potent preparation of the antihypertensive renal principle is available for trial in human hypertension.

## DISCUSSION

QUESTION.—What about the fever-producing qualities of these kidney extracts?

DR. WAKERLIN.—We studied this and have found that our renal extracts (which are administered intramuscularly) have no pyrogenic effect.

## THE RESULTS OF SURGERY IN PATENT DUCTUS ARTERIOSUS

M. J. SHAPIRO, M.D., MINNEAPOLIS, MINN.

This study was done to determine the effectiveness of surgery in patent ductus arteriosus. An analysis has been made of 643 patients who have been operated upon throughout the world. The differences in results of ligation of the duct as compared to section are determined.

Our figures show that at least 10 per cent of the cases that have been ligated have recanalized. It is also indicated that complications are more common with ligation than with section. The over-all picture is exceedingly good. The total mortality rate is approximately 5 per cent.

An analysis of our own sixty-five cases reveals that we have had the average number of recanalizations in those cases that were ligated. In approximately forty consecutive cases in our group where section has been done, there have been no fatalities. Gross, Wangenstein, and Craaford have done 172 sections of the duct with no fatalities. The follow-up study of the majority of these patients reveals the results as excellent.

It is our conclusion that surgery for patent ductus arteriosus is very effective and that the mortality rate has now been reduced to the point where all children with this lesion should be operated upon whether or not they show any evidence of cardiac strain. Surgery being more hazardous in adults, operation should be done only if such patients reveal evidence of cardiac strain, particularly if this is progressive. Patients with subacute bacterial endarteritis should be operated upon as soon as the diagnosis is made, in most instances, without attempting cure of the infection with chemotherapy. As a result of our study, we are convinced that ligation is ineffective and should be replaced by section of the duct, except in rare instances where section is not feasible.

## DISCUSSION

DR. EDWARD MASSIE, St. Louis, Mo.—I would like to ask Dr. Shapiro if he would operate upon a patient with patent ductus arteriosus if another lesion were present. We see that condition frequently and a decision has to be made as to what should be done. I sometimes hesitate in view of the presence of the other lesion.

DR. SHAPIRO.—We had one patient operated upon who had both a patent ductus and an interventricular septal defect. We predicted that the machinery murmur would disappear but that the murmur at the lower end of the sternum would persist after surgery, and this is what happened. It was important in this instance to be aware of both lesions, as it might be concluded that surgery had failed because a murmur in the heart was still present. We have another such case scheduled for surgery. This patient has a patent ductus, an interventricular septal defect, and a complete heart block undoubtedly on the basis of the defective septum. There is every reason why the patent ductus should be closed in such cases.

The question of differential diagnosis between patent ductus arteriosus and interauricular septal defect is not difficult to answer. It must be realized that patency of the duct is an

extracardiac lesion while the interauricular septal defect is intracardiac. A patent duct of any appreciable size is always accompanied by peripheral vascular changes secondary to a wide pulse pressure while an interauricular septal defect of any size produces no change in the peripheral vascular system.

## PULMONARY ARTERIOVENOUS FISTULA WITH SECONDARY POLYCYTHEMIA OCCURRING IN TWO BROTHERS: CURE BY PNEUMONECTOMY

ALFRED GOLDMAN, M.D., ST. LOUIS, MO.

This report is based on two cases of pulmonary arteriovenous fistula producing secondary polycythemia, occurring in brothers.

The first case is that of a 22-year-old man who had a long-standing polycythemia ranging up to 11,000,000 red blood cells, cyanosis, and clubbing of the extremities, all of which were known to be present since early childhood. An x-ray of the chest showed a mass in the left hilum and left mid-lung field which on fluoroscopic examination showed pulsation. There was no evidence of heart disease. The diagnosis of a cavernous angioma acting as a pulmonary arteriovenous fistula was made. A left pneumonectomy was recently performed. The lung showed a series of widely communicating channels between the pulmonary arteries and the veins, so that a good portion of the blood entering into the left lung poured directly into the pulmonary veins without oxygenation. Following the operation there was a rapid disappearance of the cyanosis, polycythemia, and clubbing of the extremities.

The second case is that of the 32-year-old brother. The red blood count was 7,000,000. There was a marked cyanosis and slight clubbing of the fingers. An x-ray of the chest showed a mass in the left mid-lung field which had a definite pulsation on fluoroscopic examination.

These two cases are the fourth and tenth cases, respectively, of pulmonary arteriovenous fistula with secondary polycythemia to be reported. This is the first time that the disease has been reported in two members of one family. A study of all ten cases shows such a characteristic and uniform picture that a clinical syndrome due to a large arteriovenous fistula can be established. A combination of polycythemia, clubbing of the extremities, a low arterial oxygen saturation, negative cardiac findings, plus an unexplained chronic pulsating pulmonary lesion is pathognomonic of a pulmonary arteriovenous fistula. Surgical removal of the lesion results in a clinical cure.

### DISCUSSION

DR. EMMET BAY, Chicago, Ill.—There was no demonstrable right heart enlargement in the first case. The large pulmonary arteriovenous fistula was shown in the pathologic specimen and I cannot figure out the mechanics of this situation. I would think the output from the right heart would have to be increased with each heartbeat.

DR. MILTON LANDOWNE, Chicago, Ill.—I should like to bring up the following two points:

(1) The tests which are used to support the diagnosis of a right to left shunt in congenital heart disease would also be expected to reveal the presence of a pulmonary arteriovenous fistula. Thus, one would expect peripheral manifestations of intravenously injected

ether (as described for congenital heart disease by Benenson and Hitzig) and possibly an apparent reduction in the "arm to tongue" depending on the size of the shunt and the amount of material used for the test.

(2) Adams, Thornton, and Eichelberger recently have reported upon the successful surgical treatment of this condition. In their case a familial nature of the disease was also suggested. They found a suggestive pulmonary shadow in the x-ray of the patient's father and in addition, their patient, his father, and possibly several other members of the family had bleeding telangiectases of the nose and mouth. I wonder whether these cases may not ultimately be classified, therefore, as a variety of Osler's familial hemorrhagic telangiectasia.

DR. GOLDMAN.—One would expect to find enlargement of the right heart as the result of a pulmonary arteriovenous fistula. However, it did not occur; this is in contrast to the situation in peripheral arteriovenous fistulas. There has been no case report in which there are any changes in the heart as a result of the fistula. This is explained by the fact that the lesion involves only the pulmonary circulation. None of the three cases that came to post mortem showed any cardiac changes attributable to the arteriovenous aneurysm.

### THE DIAGNOSTIC VALUE OF ELECTROCARDIOGRAPHIC PATTERNS BASED ON AN ASSAY OF 261 ADDITIONAL AUTOPSIED CASES

L. N. KATZ, M.D., D. FELDMAN, M.D. (BY INVITATION), AND  
R. LANGENDORF, M.D., CHICAGO, ILL.

An analysis was made of a consecutive series of 261 cases necropsied within two months after they had had an electrocardiogram made. The purpose of this study was to determine the accuracy and dependability of our electrocardiographic criteria. The thirteen specific patterns encountered are enumerated and the criteria listed. In this series forty-one cases were encountered in which discrepancy existed between the necropsy findings and the electrocardiogram; in the other 220 cases (84.3 per cent) no discrepancy was found. In general the electrocardiogram is a good index of anatomic normality and abnormality of the heart, although its use is limited by its inability to distinguish in many cases between the clinical and subclinical forms of heart disease. An abnormal electrocardiogram is excellent evidence for anatomic abnormality. A normal electrocardiogram, however, is occasionally found with an abnormal heart.

Ventricular hypertrophy is usually reflected in the electrocardiogram by heart strain patterns. Left ventricular hypertrophy more often gives rise to its strain pattern than right ventricular hypertrophy. With combined ventricular hypertrophy the pattern seen is of the predominantly hypertrophic ventricle more often than a pattern diagnostic of combined heart strain.

Recent myocardial infarctions, especially if serial records are available, are seldom missed electrocardiographically. However, old infarcts or severe coronary sclerosis without confluent infarction sometimes give rise to patterns resembling those of recent infarction. Old healed myocardial infarctions can often be diagnosed electrocardiographically but not with the regularity of recent infarction. Coronary sclerosis without myocardial infarction generally results in nonspecific electrocardiographic abnormalities.

This study confirms our previous one, making a total of 410 consecutive cases analyzed. The value of specific patterns in electrocardiography, when properly used, is thus established.

## ANTITULARENSE SERUM: CORRELATION BETWEEN PROTECTIVE CAPACITY FOR WHITE RATS AND PRECIPITABLE ANTIBODY CONTENT

LEE FOSHAY, M.D., I. RUCHMAN, PH.D. (BY INVITATION), AND  
P. S. NICHOLS, PH.D. (BY INVITATION), CINCINNATI, OHIO

Antitularense sera have hitherto failed to reveal protective antibody against challenge with strains of maximal virulence whenever the test animals were the mouse, guinea pig, hamster, or rabbit. Past inability to protect highly susceptible animals has encouraged an unfounded belief that serum therapy is therefore ineffective in the human disease. Since the reaction to tularemic infection by the rat approximates more closely that of man than do those of other animals, the rat was used for serum protection experiments.

Protective antibody was readily demonstrated in 2 c.c. quantities of each of four antitularense sera by intraperitoneal injection of the 85 to 110 gram white rat and by immediate subcutaneous challenge with 25 million viable organisms of maximal virulence, an average of 25,000 LD<sub>50</sub> rat doses.

Precipitable antibody content of sera was quantitated by the Culbertson neutralization method, using a polysaccharide prepared from acetone-extracted cells of a virulent strain by the phenol extraction method of Palmer and Gerlough.

Rat mortality was the criterion by which serum protection was judged.

Prechallenge treatment with normal sera gave 95 per cent mortality. Pre-treatment with the most potent hyperimmune serum, aged fifteen months, gave 12.5 per cent mortality. Sera of lesser potency gave correspondingly greater mortality. Mortality rates were roughly inversely proportional to precipitable antibody contents of sera. There was good agreement between antibody content and protective capacity for four immune sera of graded potencies.

The difference in clinical effectiveness between the two best sera was of the order of that shown by both rat protection and antibody content tests.

## INFLUENZA B: STUDY OF A LOCALIZED OUTBREAK PRECEDING THE 1945 EPIDEMIC\*

A. E. FELLER, M.D., CLEVELAND, OHIO

The present report is concerned with a clinical and serologic study of a localized outbreak of influenza B, which occurred in a battery of 230 recruits during the summer of 1945 at Fort Bragg, N. C. The battery had been under observation previous to the outbreak and was studied for a total period of approximately four months. Blood specimens were obtained at the beginning and end of the period of observation, thus providing the basis for correlating the clinical studies with the serologic findings.

The data showed that "recognized" and "inapparent" infections occurred with approximately equal frequency. The "recognized" infections occurred principally during a period of three weeks, indicating that a definite outbreak had occurred. However, more than half of the total infections were concentrated

\*From the Commission on Acute Respiratory Diseases.

in only one of the four platoons comprising the battery. Thus, the outbreak was localized to one segment of the population, and only sporadic infections occurred in the remainder. There was a direct but not complete relationship between low antibody titers of the initial sera and subsequent large increases in titer to influenza virus B. Also, the initial sera from those individuals who subsequently developed a febrile respiratory illness tended to have lower titers than did the sera from individuals who had either an afebrile respiratory illness or no recognized illness.

The localized nature of the outbreak seems to have been characteristic of the manner in which influenza B occurred in other parts of the country during the period of several months preceding the widespread epidemic of influenza B in the fall and winter of 1945. The fact that influenza A also occurred in a similar manner during a period of several months preceding the 1943 epidemic suggests that the sporadic or localized occurrence of influenza may indicate the imminence of an epidemic. Furthermore, the correlations between initial antibody titers and the subsequent development of serologic or clinical evidence of infection indicated that these immunologic aspects of influenza B are similar to those of influenza A.

### EVALUATION OF THE TREATMENT OF PRIMARY PNEUMOCOCCIC PNEUMONIA

ROBERT T. THOMPSON, M.D. (BY INVITATION), AND  
M. A. BLANKENHORN, M.D., CINCINNATI, OHIO

The degree to which penicillin should supplement sulfonamide drugs in the treatment of pneumococcic pneumonia is a question at the present time. Rather diverse results have been reported from various clinics so that evaluation of results is difficult. One reputable 1945 publication gives two consecutive abstracts on the treatment of pneumococcic pneumonia in which fatality rates are 18.5 and 1.1 per cent, respectively. The treatment of 189 cases of primary pneumococcic pneumonia is evaluated here in an effort to resolve apparent discrepancies in the comparison of other reports.

All of these patients were treated with sulfamerazine. A few received anti-pneumococcic serum, and a few of the later patients were given penicillin after complication developed.

Of the 189 patients treated twenty-six died. Of the 122 patients over 40 years of age twenty-four died. Of twenty-eight patients with bacteremia fourteen died. Type VII pneumococcic pneumonia occurred in thirty-five patients, and eight of these died. Of eight patients who had type VII pneumococcus bacteremia seven died.

Elevation of the blood urea nitrogen above 40 mg. per cent was found in thirty-three of the 189 patients treated. Of these thirty-three patients fourteen recovered and nineteen died.

Nineteen of the twenty-six fatal cases had azotemia prior to death. In fourteen of these it was as great, or greater, before sulfamerazine was started than at any subsequent time. Three others had azotemia before treatment which increased progressively until death. Another had mild azotemia while in shock

prior to death, and the last had a necrotizing angitis due to sulfonamide hypersensitivity which involved the kidneys, heart, liver, and spleen.

Eleven of the sixteen autopsied cases showed pathologic abnormality of the kidneys. One had hypersensitivity angitis of kidneys, two had toxic nephrosis, and eight had chronic disease of the kidneys.

Review of the fatal cases showed that shock, cardiac arrhythmia, and pulmonary edema were notable in the death of pneumococcal pneumonia patients. Every one of the sixteen autopsied cases showed pathologic abnormality of the heart; in one there was thrombotic nonbacterial endocarditis; in four there were changes of an acute toxic nature; and in eleven there were chronic or degenerative changes.

Seven patients who died were treated less than twenty-four hours.

It is concluded that delay in treatment and aggravation of latent disorder often defeat specific treatment for pneumococcal pneumonia.

#### DISCUSSION

DR. A. E. FELLER, Cleveland, Ohio.—It has been shown that pneumococcal pneumonia can be prevented, but the statement has been made that we now have such good results with sulfonamides and penicillin that it is not worth while to immunize people against this type of pneumonia. It is true that pneumococcal pneumonia does not have a high death rate or high incidence rate in certain age groups, but it seems to me that it would still be worth while to immunize against it because in many circumstances people still die from pneumococcal pneumonia as this study so clearly emphasizes.

#### A STUDY OF THE ACTION OF BETA-DIMETHYLAMINOETHYL BENZHYDRYL ETHER HYDROCHLORIDE (BENADRYL) IN THE SKIN OF HUMAN BEINGS

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ROCHESTER, MINN.

A study has been made of the mode of action, in the skin, of beta-dimethylaminoethyl benzhydryl ether hydrochloride (benadryl), a new synthetic antihistamine compound. The experiments were performed on the skin of thirty-three healthy, nonallergic persons; the size of histamine flares resulting from the standard intracutaneous injection of 1 microgram of histamine base was utilized as an indicator of the effectiveness of benadryl as an antihistamine agent. From photographic records made during each procedure, the areas of flares were accurately determined by the use of a planimeter.

Control studies performed on each person revealed that maximal flaring caused by histamine injected into skin infiltrated with isotonic solution of sodium chloride occurred during the first fifteen minutes in 90 to 95 per cent of the persons. During this time interval the maximal size of the flare in forty experiments varied from 16.1 to 24.7 square centimeters.

Histamine flares superimposed on skin infiltrated with benadryl diluted 1:1,000 were reduced 61.6 per cent in size, as compared with control flares. The intravenous administration of 150 mg. of benadryl to ten persons resulted in a 51.7 per cent reduction in size of the flare. The oral administration of 150 mg. of benadryl reduced the size of the flare only 33 per cent.

Benadryl was found to produce local anesthesia when it was injected into skin. Bio-assay, by means of electric algometric determinations, showed that benadryl in dilutions of 1:500, 1:1,000, 1:5,000, 1:10,000, and 1:20,000 possesses anesthetic potency similar to that of dilutions of procaine hydrochloride of 0.5, 0.25, 0.125, 0.0625, and 0.03125 per cent, respectively. Equivalent anesthetic doses of benadryl and procaine hydrochloride exerted definite, but not equal, antihistamine action. Procaine hydrochloride in dilutions of 0.0625 and 0.03125 per cent reduced the size of flares only 23.6 and 23.3 per cent, respectively, whereas benadryl of equal anesthetic potency, 1:10,000 and 1:20,000, caused an 80 per cent reduction in the size of histamine flares.

It was found that the pronounced antihistamine activity of benadryl is not dependent on its anesthetic potency. This was demonstrated in experiments in which injections of histamine were delayed until the anesthetic effects of benadryl and procaine hydrochloride had subsided. Flares developing in sites in which procaine hydrochloride was no longer exerting its anesthetic effect closely approximated the size of control flares. Benadryl, however, in all dilutions reduced the size of flares from 86 to 91 per cent, despite the disappearance of its anesthetic effect.

#### DISCUSSION

QUESTION.—Have you had any experience with pyribenzamine?

DR. LEAVITT.—Our preliminary experience would lead us to believe its local skin effects are very similar.

DR. CODE.—Unfortunately, even though benadryl is so potent a local anesthetic, it has irritant qualities. Other local anesthetic agents may possess antihistamine properties. State and Wangenstein used procaine in the treatment of delayed serum sickness (*J. A. M. A.* 130: 990-995, April, 1946). It is to be hoped that continued work with these and other antihistamine drugs will aid in solving some of the perplexing problems pertaining to histamine metabolism.

#### THE OCCURRENCE OF COMBINED SYSTEM DISEASE IN PERSONS WITH PERNICIOUS ANEMIA DURING TREATMENT WITH THE LACTOBACILLUS CASEI FACTOR (FOLIC ACID)

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Remissions in the macrocytic anemia of pernicious anemia, sprue, and pregnancy have been produced repeatedly with the *Lactobacillus casei* factor. This study was undertaken in November, 1945, to answer the following questions: (1) Will *L. casei* factor control the anemia of pernicious anemia and sprue for long periods of time as successfully as liver extract? (2) Will *L. casei* factor therapy prevent the development of the neurologic manifestations of pernicious anemia or control them after they have appeared? This paper presents a progress report at the end of the first year of the study.

The twenty-six subjects of the investigation were patients from the medical wards and dispensary of the Cincinnati General Hospital, twenty-one of whom had pernicious anemia which had been controlled for two to seventeen years by injections of liver extract. Three had pernicious anemia in relapse, and two had sprue which had been poorly controlled by liver extract. Each of these subjects



was treated with 70 to 105 mg. *L. casei* factor per week in divided doses given orally for ten to twelve months. All liver therapy was discontinued. Complete physical and neurologic examinations, erythrocyte counts, hemoglobin levels, hematocrit determinations, and reticulocyte counts were performed at the beginning of the study and were repeated as often as the condition of the patient demanded.

Most of the patients noted an increase in appetite and weight during the year. In only two instances were there moderate decreases in the hematologic values; these occurred after five to eight months while the subjects were receiving the same amount of *L. casei* factor which in each instance had produced a satisfactory remission previously. All manifestations of sprue were well controlled.

After five to eight months parasthesias in the extremities and an unsteady gait developed in four subjects with pernicious anemia. Two of these four subjects are mentioned previously as having developed moderate reductions in hematologic values. Neurologic signs gradually appeared and increased in numbers until there was unequivocal evidence of combined system disease. At this point each of these four patients received 50 to 500 mg. *L. casei* factor daily by mouth for ten to forty days without subjective or objective improvement. Refined liver extract, 5 c.c. intramuscularly daily, was begun after it was obvious that neurologic improvements would not occur or when progression of the disease was so rapid as to endanger the patient's future well-being. Within ten days thereafter subjective and objective neurologic improvement occurred and convalescence is progressing satisfactorily.

The conclusions are drawn that oral *L. casei* factor, 70 to 105 mg. in divided doses per week, will usually maintain patients with pernicious anemia and sprue in a normal hematologic state for a year but will not prevent the occurrence of degenerative disease of the spinal cord and peripheral nerves in patients with pernicious anemia.

### NONUTILIZATION OF CONJUGATED FOLIC ACID IN PERNICIOUS ANEMIA

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H. VICTOR NELSON, B.S. (BY INVITATION), AND ARNOLD D. WELCH,  
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The response of pernicious and allied macrocytic anemias to folic (pteroyl-glutamic) acid implies that a vitamin deficiency is corrected by this therapy. Of the folic acid occurring in foods, a large proportion consists of conjugates of higher molecular weight; thus, the conjugate of yeast (heptaglutamate) contains six additional glutamic acid molecules. Most mammalian tissues contain enzymes and conjugases that convert heptaglutamate to folic acid, and various animal species effectively utilize this conjugate in lieu of folic acid. Parenteral administration of heptaglutamate to normal man markedly augments the urinary excretion of the free vitamin.

In three patients with pernicious anemia studied, no hematologic response followed either oral or parenteral administration of the conjugate, but a definite response occurred in two of these to whom an equivalent amount of folic acid

was given subsequently. Furthermore, in contrast to the finding in normal man, administration of heptaglutamate to the three patients studied caused little or no augmentation of the urinary elimination of folic acid. Bethell and associates have independently observed the ineffectiveness of heptaglutamate in several patients with pernicious anemia.

Although these findings may offer a basis for the apparent folic acid deficiency of pernicious anemia, a conclusion that the utilization of heptaglutamate is due to a direct action of the antipernicious anemia factor is at present unjustified. Augmentation of the conjugase activity of human bone marrow *in vitro* has appeared to result from the addition of highly purified liver extract, but the results are obtained inconsistently and are of uncertain significance. Conceivably, liver extracts contain a factor necessary for the proper functioning of a conjugase system or a substance concerned with the removal of an inhibitor of a conjugase system. However, the ineffective utilization of the naturally occurring conjugate by patients with pernicious anemia may be secondary to a more deep-seated biochemical derangement.

In favor of this view is the observation that the activity, in patients with pernicious anemia of those conjugase systems studied, is not significantly less than that of normal subjects. Also, the striking effectiveness of purified liver extract in one patient in whom folic acid was almost ineffective, in intramuscular doses up to 12 mg. daily, suggests that the factor(s) of purified liver extracts, although apparently concerned in some way with the release of folic acid from its conjugate, in addition has other functions.

#### FURTHER STUDIES ON THE UTILIZATION OF PTEROYL HEXA- GLUTAMYL GLUTAMIC ACID (VITAMIN B<sub>12</sub> CONJUGATE) IN PERNICIOUS ANEMIA

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BROWN, PH.D. (BY INVITATION), DETROIT, MICH., MURIEL C. MEYERS,  
M.D., AND GOULD A. ANDREWS, M.D., ANN ARBOR, MICH.

The effectiveness of pteroylglutamic acid\* (P. G. A.) in the treatment of certain macrocytic anemias raises the question of the cause of a deficiency of this substance in such anemias. Although widely distributed in common foods, this component of the vitamin B complex is usually present as a conjugate, pteroyl hexaglutamyl glutamic acid. Consequently an apparent deficiency of P. G. A. in persons whose diets have not been grossly inadequate suggests a metabolic defect in the utilization of the natural material. Evidence has been obtained of such a defect by observations of hematologic responses and by determinations of the urinary excretion of P. G. A. in patients with anemia after the oral administration of the vitamin in the conjugated and in the free form. In this communication the results of earlier studies as well as additional investigations on this problem will be reported.

The hexaglutamyl conjugate was given orally in the form of a yeast concentrate to fourteen patients and to six healthy subjects. There were nine

\*This is the chemical name of the vitamin which has been referred to as folic acid, vitamin M, or vitamin B<sub>12</sub>.

patients with pernicious anemia in relapse, three with pernicious anemia in liver extract induced remission, and two with macrocytic anemia following total or subtotal gastrectomy. Doses of conjugate supplying the equivalent of 4 to 5 mg. of crystalline P. G. A. daily were without effect on the erythrocytes, reticulocytes, hemoglobin, or hematocrit values of any of the patients with anemia. The administration of equal amounts of the vitamin in the free form (Folvite\*) was followed in all instances by significant hematologic increases, usually approximating the expected results from adequate oral liver extract or stomach therapy.

The P. G. A. content of the urine was determined by microbiologic assay. No evidence for the excretion of the vitamin as conjugate was obtained in any of the experiments. The excretion of the vitamin while the subjects received unsupplemented diets was generally lower in the case of the patients with macrocytic anemia than in the case of the normal persons.

After the administration of the vitamin conjugate the patients with macrocytic anemia showed no significant increase in excretion of P. G. A., with the exception of one subject who excreted slightly greater amounts, whereas the normal individuals excreted relatively large quantities. After the administration of free vitamin the patients excreted variable amounts, whereas the values for the normal subjects were considerably higher and more constant.

Administration of conjugate to three patients with pernicious anemia who had been receiving parenteral liver extract for a number of years and whose blood values were within normal limits was followed by excretion of the free vitamin in amounts comparable to those excreted by normal persons.

It is believed that these results demonstrate that the normal person can convert the vitamin conjugate to the free form and that in untreated pernicious anemia this ability may be either greatly reduced or entirely lacking.

It seems probable that for P. G. A. to participate in hematopoiesis (megalo-blastic maturation) the vitamin must be present in the free state.

The difference in excretion levels of the vitamin after administration of the conjugate, between patients in relapse and those in liver extract-induced remission, suggests that the principle of liver active in pernicious anemia may be concerned with the conversion of the conjugated vitamin to the free form.

#### DISCUSSION

DR. BYRON E. HALL, Rochester, Minn.—During the past year observations similar to those just reported by Dr. Vilter and his associates have been made at the Mayo Clinic; namely, that administration of synthetic folic acid neither protects the patient with pernicious anemia from neurologic manifestations nor relieves him of them once they have developed. In our series of fourteen patients with pernicious anemia who were treated for many months by administration of synthetic folic acid, ten instances of peripheral neuropathy as manifested by paresthesia and two of early subacute combined degeneration of the spinal cord were observed before institution of treatment. Of the ten instances of paresthesia improvement occurred in four, temporary improvement followed by relapse occurred in four, and no improvement was observed in two. In the two instances of subacute combined degeneration of the spinal cord which were observed before institution of treatment, evidence of progression of the neurologic manifestations of the disease was noted during administration of synthetic folic acid. In the cases in which symptoms or signs of neurologic involvement did not exist prior to the institution of treatment with folic acid, two instances of paresthesia and three of early

\*Lederle Laboratories, Inc., Pearl River, N. Y.

signs of combined sclerosis occurred while treatment was being given. Neurologic manifestations developed in some cases despite induction of satisfactory hematologic remissions.

Another observation has been that of the wide variation in rapidity of hematologic response of patients with pernicious anemia in relapse who were treated with synthetic folic acid. While an occasional patient responds as rapidly to treatment with folic acid as to treatment with liver, the majority respond hematologically more slowly to treatment with folic acid. The response of three of fourteen patients treated with folic acid was very slow and hematologic remissions could not be induced despite administration of fairly large doses of the preparation; however, satisfactory hematologic remissions were induced in all three patients by means of intensive treatment with liver.

The appearance of sternal marrow obtained by serial aspiration in our group of cases has indicated that synthetic folic acid rapidly converts erythropoiesis from a megaloblastic to a normoblastic type. While the effect of this agent on the bone marrow and blood of patients with pernicious anemia in relapse is of great interest, it is obvious that the substitution of synthetic folic acid for liver in treatment of pernicious anemia is extremely hazardous in view of the demonstration that administration of folic acid does not prevent development of neurologic manifestations of the disease.

DR. WALTER L. PALMER, CHICAGO, ILL.—The first two papers and the discussion by Dr. Hall all suggest that folic acid is not a complete substitute for liver extract. It may well be that this is true, but we should be very careful in arriving at this conclusion. I am reminded of the experience we all have had with liver through the years. At first we thought it was not satisfactory in all cases; then we thought the crude form was better than the refined; and finally we decided that refined liver extract was a complete substitute for whole liver and for the crude extract as well. The problem seemed to resolve itself into (1) the method of administration and (2) the dosage. In the first paper the route of administration of the folic acid was oral. We all know that the oral method of administration of liver extract is very unsatisfactory in many instances and that there are some patients who cannot be maintained with normal blood pictures in this manner. With regard to dosage, apparently the largest amount of folic acid given intramuscularly was 12 milligrams. This may not be enough. Would it not be worth while to try 50 mg. per day intramuscularly?

DR. CECIL J. WATSON, Minneapolis, Minn.—I want to ask whether in the urinary excretion studies the results were based on bio-assay technique and whether any studies on conjugated or free folic acid have been made on the feces of these patients.

DR. HOWARD L. ALT, Chicago, Ill.—I would like to ask about the diet these patients received who had relapses of cord disease on folic acid treatment.

DR. M. W. COMFORT, Rochester, Minn.—It might be worth while at this time to mention the disappointing results obtained so far in the treatment of nontropical sprue with synthetic folic acid. This is in contrast to the excellent response of tropical sprue to treatment with the same preparation. My colleagues and I have treated a number of patients who have nontropical sprue with adequate doses of synthetic folic acid for several months. Four patients received 50 to 200 mg. of folic acid a day intramuscularly, and the results to date are as follows: Diarrhea has not been significantly affected and relapses have continued to occur with about the same degree of frequency. In one patient in whom megaloblastic bone marrow was present the bone marrow changed rapidly to a normoblastic type. In all four patients the reticulocytes have responded slightly, but so far the percentage has not exceeded 4. In none of these patients has the size of the erythrocytes been reduced or has the anemia improved to a satisfactory degree. One patient is dead apparently as the result of an exacerbation of the disease, and two are in exceedingly serious condition. Of the patients given folic acid by mouth, changes have not been seen in their condition that could not be ascribed to the fluctuations in the severity of the disease. These disappointing results suggest that in some patients nontropical sprue will not respond favorably to treatment with synthetic folic acid.

DR. R. W. VILTER, Cincinnati, Ohio.—Regarding Dr. Palmer's comments, in working up this paper for presentation I went back over the experiences from 1929 to 1935 with liver extract in this same type of patient. There was considerable divergence of opinion, as

Dr. Palmer indicated, as to the effectiveness of liver extract in combined system disease of pernicious anemia. From the literature I was unable to tell why there was this divergence of opinion because most of the liver extract was given intramuscularly in adequate doses, so I could not come up with any analogy between their experience at that time and our experience now with folic acid. Generally speaking folic acid given by mouth is almost as effectively absorbed and utilized as folic acid given intramuscularly. The average effective dose is usually 2 to 5 mg. by the parenteral route or 10 to 15 mg. by oral route. In contrast, liver extract is poorly absorbed and utilized by the oral route, the effective oral dose being many hundred times the intramuscular dose. The interesting thing regarding the diets of the patients who relapsed is that patients whom we knew were on deficient diets did not develop neurologic symptoms, whereas patients in the private group who were referred because of sensitivity to liver developed neurologic manifestations. The diets of the patients in the private group were much better than those of the patients from the dispensary. I do not believe that diet had much to do with these relapses.

As to Dr. Comfort's comments, I have a feeling that nontropical sprue is a group of diseases having poor intestinal absorption and steatorrhea as a common denominator rather than a single disease. Folic acid will not benefit patients who appear to have sprue but in reality have some other disease, for instance lipophagic granulomatosis. The two patients I mentioned were from Cincinnati and would be classified as nontropical sprue; they both fared better on folic acid than they had in five years on liver extract.

DR. ROBERT W. HEINLE, Cleveland, Ohio.—We have forty patients with pernicious anemia who have been under treatment with folic acid for from seven to nine months. They had received liver extract for many months or years before folic acid was given. In the short period they have been on folic acid, none of them has shown neurologic relapse. I do not want you to think that I am overenthusiastic about this drug. I do not think it has been proved to be as good as liver extract, but I do agree with Dr. Palmer that we have not tested it out thoroughly, particularly as to the route of administration and the dosage.

Dr. Palmer criticized the case of the one patient to whom we gave 12 mg. daily. His is a valid criticism, but that patient had been on an experimental regimen for fifty-two days without benefit. Other patients on that dose had received more benefit. Whether this one patient would have received more benefit if the folic acid had been given in larger doses I do not know.

The method of assaying conjugated folic acid in the urine is the standard method utilizing *Lactobacillus casei*. We have attempted to make these same studies on feces. This is difficult because of the high bacterial content. We are working on that problem at present and our preliminary study would indicate that very little folic acid is present in the stool.

With reference to Dr. Comfort's remarks about nontropical sprue, I have had a limited experience. Tremendous doses of liver seem to be necessary to obtain satisfactory results in many cases. Intravenous administration of crude liver extract has been recommended. The feeling has been that intravenous doses of crude liver extract in large amounts may be better than the administration of purified liver extract.

There is one thing I would like to say about the clinical use of folic acid. The dose schedule recommended by the manufacturer is too low. They recommend daily doses of 5 to 20 mg. or intramuscular doses of 15 mg. daily. It is possible that some patients can be brought into remission on that dose, but it is more likely that a great many will need larger doses than this, and I do not think we should pay too much attention to the recommended schedule. It should be noted that the manufacturers of folic acid suggest that better results may be obtained by the simultaneous administration of folic acid and liver extract than with folic acid alone.

### RUTIN IN PURPURA

F. W. MADISON, M.D., AND H. W. POHLE, M.D. (BY INVITATION),  
MILWAUKEE, WIS.

It is probable that one of the etiologic mechanisms capable of producing the vascular fragility characteristic of purpura is of deficiency type. For many

years avitaminosis C was considered to be of major importance, but recent studies have suggested that it is much less frequently a factor than was previously supposed. In 1936 Szent-Györgi observed that citrin derived from paprika or lemon was capable of reversing experimentally produced vascular fragility. He designated it as vitamin P and suggested that deficiency of that substance was responsible for purpuric changes in some instances. Two crystalline flavone glucosides derived from citrin, hesperidin, and eriodictol have failed to produce the same effect noted in the use of citrin. Recently a flavonol glucoside, rutin, closely related to hesperidin, has been used in a similar manner and has seemed to be more effective than either of the flavone glucosides. It has been used in 173 reported cases of purpura with reversal of fragility in 152 cases (88 per cent).

Our clinical experience with rutin has been limited by the supply available, but the results thus far have been of such nature as to suggest that it does exert a favorable influence on vascular fragility in some types of purpura. Its effect has been studied in fourteen cases of clinical purpura, six of which were of probable allergic origin, two toxic, one endocrine, one associated with hypertension and retinal hemorrhages, and four associated with advanced malignant disease. The most striking results were obtained in the last group where cessation of the drug resulted in relapse and resumption in remission. Less convincing results were seen in the allergic group and questionable results in the remaining groups.

### THE EFFECT OF TOTAL BODY X-IRRADIATION ON A PRE-EXISTING INDUCED ANEMIA

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ERIC L. SIMMONS, PH.D. (BY INVITATION), CHICAGO, ILL.

One of the problems facing those of us charged with the responsibility of the protection of personnel from radiation hazards on the plutonium project was whether individuals with a pre-existing anemia would be more adversely affected by externally originating total body irradiation than individuals with normal values. In an attempt to study this problem, a regenerative anemia was produced in rabbits by the subcutaneous administration of phenylhydrazine hydrochloride. The hematologic effects of 800 r. total body x-irradiation on such anemic animals were compared to effects on animals given only phenylhydrazine and allowed to recover spontaneously and to a group of animals given 800 r. alone. A group of normal untreated animals was studied simultaneously.

The characteristic initial rise and subsequent reduction in heterophils and immediate precipitous fall in the lymphocyte values followed the administration of 800 r. in the group in which a regenerative anemia had been produced with phenylhydrazine as well as in normal control animals. The time of recovery of those elements to normal limits in the two groups was likewise comparable. An "abortive rise" in the leucocyte values of both of these groups was noted between the fifth and eleventh days after the irradiation.

The group of animals which received x-irradiation alone developed an anemia with its maximum at fourteen days and with recovery by twenty-three days. The animals in which a regenerative anemia was produced by phenyl-

hydrazine and which were irradiated at or near the point of maximum anemia developed no further anemia, values returning to normal limits by twenty-three days. The erythrocyte and hemoglobin values of the group in which an anemia was produced by phenylhydrazine and which were allowed to recover spontaneously, however, returned to normal within fourteen days.

The reticulocyte values of animals which received phenylhydrazine had reached approximately 21 per cent when the x-irradiation was given and were reduced to a minimum value of 1.0 per cent within two days after the irradiation. Recovery to normal limits occurred by nine days in this group. The reticulocyte values of normal control animals which were given 800 r. fell maximally within two days, reached a minimum of less than 0.1 per cent, and recovered slowly but completely by twenty-three days.

The failure of 800 r. total body x-irradiation to produce further anemia in animals with a previously induced regenerative anemia or to materially interfere with recovery is dependent in part on the number of surviving erythroblasts in the erythropoietic tissue.

Histologic studies made on animals prepared in a comparable manner and sacrificed at varying appropriate intervals indicate that erythropoietic tissue was completely destroyed by seventy-two hours in the normal rabbits which received 800 r. and recovery proceeded slowly. On the other hand, although massive destruction of erythropoietic tissue occurred after exposure to 800 r. in animals in whom a regenerative anemia had been produced with phenylhydrazine, this destruction was delayed beyond seventy-two hours. This appears to indicate that a latency to x-ray effect was produced in erythropoietic tissue which had previously been stimulated.

## ON THE RELATION OF CALCIUM TO THE ACTIVATION OF PROTHROMBIN AND ITS SIGNIFICANCE IN DICUMAROL POISONING

ARMAND J. QUICK, M.D., MILWAUKEE, WIS.

The role of calcium in the coagulation mechanism has not been satisfactorily defined, and the generally accepted concept that prothrombin is activated by thromboplastin in the presence of ionic calcium has failed to advance knowledge concerning the chemical action of calcium in its participation in coagulation. The recent finding (Steinberg) that a phenol-formaldehyde resin known commercially as Amberlite can quantitatively remove calcium from blood, without producing any other demonstrable change, offers a new means for studying quantitatively the calcium requirement for coagulation and specifically for the activation of prothrombin.

It was found that a fixed amount of calcium is required for the optimum activation of prothrombin, that is, for the shortest prothrombin time. For human plasma it is approximately 0.0012 M. and for dog blood 0.0004 M. Higher concentrations have no further effect except that a depressive action begins when the calcium ion concentration exceeds 0.02 M. Below the optimum or critical concentration of calcium, the prothrombin time becomes progressively delayed. Since this critical level is below the concentration of the

free or ionized calcium, it appears that ionized calcium is not involved in coagulation. One can conjecture that plasma contains a calcium cofactor which with thromboplastin and calcium becomes the activator of prothrombin. Evidence was obtained that this cofactor is not directly dependent upon either component A or B of prothrombin.

When Dicumarol was fed to dogs, the minimum or critical concentration of calcium required for optimum activation of prothrombin increased as the prothrombin (or more accurately component B) decreased. When the prothrombin time became twenty seconds, the normal concentration of the calcium of the blood was no longer sufficient for optimum prothrombin activity. This suggests that the delayed coagulation in dicumarol poisoning may not be solely the resultant of prothrombin depletion but may be influenced by an inadequate concentration of calcium. On the basis of these results, the unreliability of the bedside method for the determination of prothrombin becomes obvious, since in this test thromboplastin without additional calcium is added directly to blood.

#### DISCUSSION

DR. CHARLES F. CODE, *Rochester, Minn.*—I should like to ask if Dr. Quick has tested the effect of changes in the concentration of prothrombin upon the optimum calcium concentration in an artificial setup using purified constituents in which the prothrombin concentration might be varied at will. I wonder if a shift in the amount of prothrombin alone would alter the optimum calcium concentration.

DR. KENNETH M. BRINKHOUS, *Iowa City, Iowa.*—Since amberlite is very effective in removing electrolytes, I wonder if you have data on the concentration of other plasma electrolytes in the amberlite-treated plasma. This factor might have had an effect on some of these results, as electrolyte concentration is known to influence considerably the rates of several of the clotting reactions.

DR. QUICK.—In regard to Dr. Code's question about an artificial setup, we have had no experience. This work is still preliminary and we need to do much more on it. Probably we can set up certain experiments to get further information.

In answer to Dr. Brinkhous' remarks, we use amberlite treated with saline and so far as we know the action is primarily an exchange one, an atom of sodium for one of calcium. It gives off one atom of sodium and takes up one of calcium. There may be other changes, and probably the action is not as simple as that, but in our work we found no evidence of action other than the one of exchange.

#### MORPHOLOGY OF BONE MARROW

IMPRINT AND HISTOLOGIC PREPARATION OF ASPIRATED STERNAL MARROW UNITS  
E. M. SCHLEICHER (BY INVITATION), AND E. A. SHARP, M.D., DETROIT, MICH.

It has been established that direct and indirect (heparinized and centrifuged) bone marrow spreads and differential counts made within a measured area offer a means of distinguishing morphologic differences as well as quantitative relationships.

At the same time these methods fail in two important features, namely, (1) the preservation of cellular cyncytia which permit a study of marrow cell development and (2) maintaining intact the histologic pattern of sternal marrow units. These latter configurations are essential in the diagnosis of early infectious or malignant processes of this hemopoietic organ.



It is the purpose of this paper to present representative examples of "imprint" and intact "histologic" preparations of sternal marrow in black and white and kodachrome, showing the methods of preparation and the normal compared with the pathologic morphology. It will be demonstrated that maturation arrest in pernicious anemia does not occur at the megaloblastic (pronormoblastic) stage but rather that the reticulum is pathologically activated. Metastatic carcinoma, tuberculosis, and various patterns in blood disorders will be presented.

## THE EFFECT OF RADIATION THERAPY ON THE NOCTURNAL GASTRIC SECRETION IN PATIENTS WITH DUODENAL ULCER

ERWIN LEVIN, M.D. (BY INVITATION), WALTER LINCOLN PALMER, M.D.,  
AND JOSEPH B. KIRSNER, M.D., CHICAGO, ILL.

The fasting nocturnal gastric secretion was studied in twenty-eight patients with active uncomplicated duodenal ulcer. The twelve-hour volume ranged from 550 to 1,750 c.c. with an average of 1,110 cubic centimeters. The free acidity varied from 18 to 122 clinical units, the average being 62. The night secretion was studied in nine of these patients during the period they received x-ray therapy directed at the fundus and body of the stomach. A reduction in volume ranging from 6.3 to 51.6 per cent was obtained in seven patients during the first week of therapy. In two patients an increase in volume was noted. A reduction in free acidity was observed in seven patients. Nocturnal gastric secretion was studied in fifteen patients of the group at varying intervals after therapy. A decrease in volume occurred in fourteen patients, the average reduction being 52.1 per cent. In one patient the volume was increased but the free acidity disappeared. There was a marked reduction in the free acidity of the nocturnal secretion in all patients, the reduction amounting in thirteen of the fifteen to anacidity. A comparable reduction was observed in the gastric secretory response to histamine. These studies indicate that radiation therapy decreases the gastric secretion as measured in the fasting state and by the response to histamine. The duration of the effect is variable.

### DISCUSSION

DR. LEON SCHIFF, Cincinnati, Ohio.—I would like to ask how your results in the reduction of volume and acidity of gastric contents compare with those seen following bilateral vagotomy.

DR. MOSES BARRON, Minneapolis, Minn.—I would like to ask if any other method than x-ray was used.

DR. GEORGE WAKERLIN, Chicago, Ill.—Were pepsin studies made on these patients and, if so, what were the results?

DR. LEVIN.—In answer to Dr. Schiff's question regarding the reduction of gastric secretion following x-ray therapy compared to the reduction following vagotomy, of course the effect of x-ray therapy is not permanent. Some patients, I have been told, have had a reduced gastric content for two or three years. It is still too soon to predict what the results of vagotomy will be. I had the opportunity to study one patient who had had vagotomy three years previously. His volume, free acid, and night secretion are still reduced, and there is no free acid in the night secretion.

As to the medical management, all of these patients were on Sippy management. While the studies were being carried on, no patient received any drug that would depress gastric secretion.

Pepsin studies are now being carried out in an effort to determine if that is reduced by x-ray therapy. I am told that there is some reduction in peptic activity.

## THE MEASUREMENT OF HUMAN GASTRIC FUNCTION

ARTHUR M. SCHOEN, M.D. (BY INVITATION), AND P. K. KNOEFEL, M.D.,  
LOUISVILLE, KY.

Past studies of human gastric function have been concerned largely with the aspiration of gastric contents and the measurement of volume and composition. The actual rate and nature of the secretion cannot be determined from these two observations alone, since gastric emptying occurs. Because of this, others have injected foreign substances into the stomach, such as readily determinable dyes, and have determined more accurately the composition of gastric juice but not the rate of secretion. It is possible to determine the secretory rate and emptying rate if a volume of isotonic NaCl solution ( $V_o$ ) containing phenol red ( $W_o$ ) is injected into the empty stomach and the residual contents of the stomach ( $V_t$ ) containing phenol red ( $W_t$ ) are aspirated at time ( $t$ ). Then the volume secreted ( $S$ ) in time ( $t$ ) is

$$S = (V_o - V_t) \left\{ \left[ \text{Log} \left( \frac{W_t}{W_o} \right) \div \text{Log} \left( \frac{V_t}{V_o} \right) \right] - 1 \right\}$$

and the volume emptied ( $E$ ) is

$$E = S - V_t + V_o.$$

The necessary assumptions are: (1) No gastric absorption of water or phenol red occurs, (2) no saliva or duodenal contents enter the stomach, (3) there is uniform distribution of phenol red throughout gastric contents during time ( $t$ ), (4) secretion and emptying occur at constant rates throughout time ( $t$ ), and (5) gastric contents are completely aspirated at time ( $t$ ). A satisfactory technique for obtaining gastric juice was developed. This includes preparation of patient for basal conditions, selection and preparation of gastric tube, insertion of tube and determination of location by fluoroscopy, and procedure for complete aspiration. Evidence for the correctness of these assumptions and the validity of the method has been obtained.

From the previously mentioned data and the measured pH and peptic activity of gastric contents, the pH and peptic activity of gastric juice as secreted can be calculated. The resting rate of gastric secretion seen in forty observations in twelve normal persons was 2.09 c.c. per minute, standard deviation 0.96. In one person the daily average basal secretory rate on five days varied from 2.07 to 4.33 c.c. per minute. The average deviation in consecutive observations in the same person was 12.4 per cent. The average hydrogen ion concentration of the secretion was pH 1.51 in twenty-six observations. Studies have been made in patients with peptic ulcer during remissions and exacerbations and in a variety of conditions as aplastic anemia, cholecystitis, pancreatitis, and neurosis. The

responses of normal persons and patients with ulcers to sham feeding, antacids, alcohol, caffeine, histamine, atropine, and insulin have been studied before and after vagotomy.

#### DISCUSSION

DR. WALTER L. PALMER, Chicago, Ill.—Dr. Schoen and Dr. Knoefel have certainly made gastric analysis scientific. The fallacies of the older methods have been recognized and apparently corrected in this excellent contribution. I wonder if the essayist can answer the question of whether there is a higher level of gastric secretion in a patient with duodenal ulcers than in a normal individual.

DR. SCHOEN.—We have made 161 of these tests. We hesitate to do the work on people who have active duodenal ulcers. We have done some on such patients and those tests have shown definitely that with healing of the ulcer there is a decrease in the rate of gastric secretion.

### THE EFFECT OF ENTEROGASTRONE ON FASTING GASTRIC SECRETION AND MOTILITY IN PATIENTS WITH DUODENAL ULCERS

H. MARVIN POLLARD, M.D., MALCOLM BLOCK, M.D. (BY INVITATION), AND  
W. H. BACHRACH, M.D. (BY INVITATION), ANN ARBOR, MICH.

Enterogastrone has been reported to be effective in the prevention of jejunal ulcers in Mann-Williamson dogs and the prevention of recurrence of peptic ulcer in human beings. The possible mechanisms of its effect are (1) an inhibition of secretion of hydrochloric acid, (2) an inhibition of motility, and (3) an increased resistance of the gastric and duodenal mucosa to ulceration. It has been reported that Mann-Williamson dogs treated for several months with enterogastrone do not exhibit the excessive gastric secretory response to alcohol characteristic of most untreated Mann-Williamson dogs; furthermore, urogastrone has been shown to inhibit the histamine-stimulated gastric secretion in normal human beings. Studies of the effect of enterogastrone on human motility have not been reported.

Our preliminary findings on three patients with intractable duodenal ulcers treated for at least three months with enterogastrone orally show no consistent reduction in the fasting output of hydrochloride by the stomach.

Simultaneous motility and secretion studies after a parenteral dose of 200 mg. of enterogastrone show only a transitory inhibition of motility and secretion. The effect of large doses has not yet been worked out.

The clinical course of the three treated patients has not been followed for a sufficient period of time to evaluate the therapeutic effects of the preparation.

The studies are being continued.

#### DISCUSSION

DR. JOSEPH B. KIRSNER, Chicago, Ill.—We have been interested in this problem of the effect of enterogastrone on gastric secretion in man. We have not had the oral preparation available. After studying a relatively large number of patients, using larger quantities of the parenteral preparation, we can confirm some of the observations of Dr. Bachrach; that is, the effect of enterogastrone in large doses is rather variable and unpredictable. In certain cases there is a pronounced decrease in the volume and level of gastric acidity even to the

absence of hydrochloric acid in the gastric content. One of the interesting things is that this effect may not become apparent for twenty-four to thirty-six hours after the onset of treatment. In our studies the tube has been kept in place for several days.

Another point I would like to make is that this effect of enterogastrone on gastric secretion is not a fixed one. In one patient with duodenal ulcers, during the course of the experiment an achlorhydria resulted, but during a disturbing psychiatric interview with the patient there was a marked rise in volume of secretion and in the level of hydrochloric acid. Later, after the interview had ended and enterogastrone was continued, there was again a drop in the volume of gastric secretion. This observation illustrates what I am sure is now well known, namely, the effect of the emotions on gastric function.

Dr. Bachrach mentioned an important point with regard to the local pain following the injection of this product. We have found this to be true, and on one occasion, after giving a large dose, the patient continued to be distressed ten days after the injection.

DR. BACHRACH.—We had achlorhydria in two patients, but they both had gastric ulcers. They got well without the benefit of enterogastrone.

## EFFECTS OF ALLOXAN UPON THE HUMAN PANCREAS

JEROME W. CONN, M.D., D. L. HINERMAN, M.D. (BY INVITATION), AND  
R. W. BUXTON, M.D. (BY INVITATION), ANN ARBOR, MICH.

It has now been established that a single dose of alloxan, varying from 50 to 400 mg. per kilogram, produces destructive changes in the pancreatic islets of the dog, monkey, pigeon, duck, guinea pig, cat, and rat. When administered in smaller daily doses a much larger total amount is required to produce evidence of islet cell damage. An occasional animal is found to be refractory.

The only reported observations of the effects of alloxan upon human islet tissue are by Brunschwig and associates (two cases). No histologic changes in the islet tissue were found (1) seven hours after a single injection of 600 mg. per kilogram and (2) one month after a total of 3,150 mg. per kilogram given irregularly over a fifty-day period. The impression has been gained, therefore, that human islet tissue is exceedingly resistant to the damaging effects of alloxan.

Our study was done on a patient harboring a secreting pancreatic insuloma. On each of nine consecutive days she was given alloxan intravenously in the following doses in milligrams per kilogram: 50, 50, 100, 100, 100, 100, 100, 100, 100. Fifteen days after the last injection an islet cell tumor was removed surgically and a section of pancreas was obtained for study.

Microscopic examination of the tissue, stained differentially by several methods, shows no recognizable effect of alloxan upon the islet cells of the neoplasm. However, clear evidence of marked destruction of the islets of the pancreas proper and of early repair are observed. The pathologic changes seen in these islets are typical of those which occur in alloxan-treated animals and resemble most closely those described by Baily and associates as occurring in rabbits given small daily injections for from seven to thirteen days.

The metabolic studies complement the pathologic findings. In the 15-day interval after alloxan and before operation, the insuloma continued to secrete insulin as indicated by the continuation of an extremely low daily level of the fasting blood sugar. In this same period, however, and despite the low fasting blood sugar, a marked decrease in carbohydrate tolerance was apparent. Upon removal of the tumor frank diabetes developed requiring large amounts of

insulin despite low carbohydrate intake. Insulin was discontinued on the forty-fifth postoperative day. To date (sixty-five days postoperative) carbohydrate tolerance has improved but mild diabetes persists.

### *Conclusions.*—

1. In this study the sensitivity of human islet tissue to the destructive effects of alloxan approximates that observed in other species.
2. Neoplastic islet tissue is much more resistant to alloxan than is normal islet tissue.
3. In future attempts to use alloxan therapeutically these facts should be considered.

### DISCUSSION

DR. GEORGE GOMORI, Chicago, Ill.—I had a patient dying of carcinoma of the breast with metastases. She was given 500 mg. of alloxan per kilogram of body weight and died thirteen hours later. Autopsy showed no trace of change in the islands of Langerhans. I wonder whether changes shown here cannot be due to massive intravenous infusions of glucose. I have seen a number of specimens from patients who had to be treated with massive doses of glucose. In three or four of these cases the changes were very much like the ones shown here, although the patients did not receive alloxan. Similar degenerative changes are occasionally seen also in animals treated with large doses of insulin. The changes presented here also may be due to massive amounts of insulin released by the tumor.

DR. CONN.—This patient did not receive intravenous glucose therapy in the preoperative period. In attempting to settle the problem of whether hyperinsulinism, per se, may produce similar islet cell changes, we are carrying out long-term experiments in dogs. Preliminary evidence indicates that normal dogs given sufficient protamine insulin daily to keep the fasting blood sugar in the same range as this patient's had been show no islet cell changes similar to those seen in the patient following the course of alloxan.

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## BOOK REVIEWS

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**Medical Jurisprudence and Toxicology.** Eighth edition. By John Glaister, J.P., D.Sc., M.D., F.R.S. (Ed.), Of the Inner Temple, Barrister-at-Law, etc., Regius Professor of Forensic Medicine, University of Glasgow; formerly Professor of Forensic Medicine, University of Egypt, Cairo; and Medico-Legal Consultant to the Egyptian Government. Williams & Wilkins Company, Baltimore, 1945. Price \$8.00. Cloth with 222 illustrations and 691 pages.

This well-illustrated book is intended to cover the main aspects of Forensic Medicine. It is divided into two parts. The first part deals with medical jurisprudence and describes the methods used to determine the cause of death produced by asphyxia, drowning, burning, electricity, hanging, exposure to cold, or by homicidal injuries. Furthermore, injuries produced by firearms, the identification of blood and seminal stains, and the medico-legal aspects of sex crimes are reviewed. The second part of the book is concerned with toxicology. The etiology, the clinical signs, and the treatment, as well as the post-mortem appearances, and the chemical diagnosis of poisonings are described.

The usefulness of the book in this country is limited by the fact that it considers exclusively the British laws. The clinical aspects of toxicology are adequately presented, but many chemical tests described lack specificity or sensitivity and appear to be of relatively little utility in the detection of poisons in biological materials.

ERNEST BUEDIG, M.D.

# RAPID IDENTIFICATION OF SHIGELLA IN A PUBLIC HEALTH LABORATORY

W. W. FERGUSON, PH.D., MARY BRANSTON, A.B., GRACE L. MCCALLUM, B.S.,  
AND MARGARET J. CARLSON, B.S.  
LANSING, MICH.

BOYD'S concept of the antigenic structure of Flexner dysentery bacteria has been given practical application in this country by Wheeler.<sup>1</sup> He<sup>2</sup> recognized that the essential differences between Flexner types of *Shigella* are the specific antigens, while the group components are of secondary importance, except perhaps in the differentiation of Flexner W into subtypes. Wheeler<sup>1</sup> has described methods for serologic identification of other pathogenic *Shigella*, as well as the Flexner and Boyd types,\* by means of a slide agglutination test employing formalinized antigens and properly absorbed sera. He has specified, however, that "classification of the organisms as determined by the agglutination test should be confirmed by fermentation reactions."

Weil and associates<sup>3</sup> are in apparent agreement with Boyd and Wheeler that specific or "primary" antigens distinguish the Flexner as well as the Boyd types from each other. However, some of the dual antigens recognized by Weil and co-workers would be regarded, necessarily, as group antigens by Wheeler. In effect, the former authors have combined the concept of Boyd with that of Andrewes and Inman.<sup>4</sup>

Weil and associates<sup>3</sup> have advocated the use of absorbed sera for rapid identification of *Shigella*. More recently, Weil and co-workers<sup>7</sup> have established the existence of minor antigens which interrelate *Shigella ambigua*, *Shigella alkalescens*, and *Shigella dispar* with certain of the Boyd types. They caution that species (and type) identification must rest on both immunologic and cultural properties.

Nelson and associates,<sup>8</sup> who examined 450 "presumptively positive" enteric cultures in India, feel that the use of absorbed sera in the spot agglutination test for identification of *Shigella* is far superior to the use of unabsorbed sera in the conventional tube agglutination method. Eighty per cent of the cultures examined by these investigators were identified as *Shigella*, presumably by serologic means alone.

Extensive use of *Shigella* typing sera was made in Army laboratories in various parts of the world during World War II. Outside of one brief publication,<sup>9</sup> no information on the sera and their uses has appeared in the literature.

From the Bureau of Laboratories, Michigan Department of Health.

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\*Throughout this paper the Flexner types will be designated as Flexner V, W, X, Y, and Z and the Boyd types as 103, P119, 85, 170, P285, D1, D19, P274, and P143. At present the different systems for designating Flexner and Boyd types, as proposed by Boyd,<sup>1</sup> Wheeler,<sup>2</sup> and Weil and associates,<sup>3</sup> are in disagreement. The effect on the literature of the various systems may be judged from the following example. Francis<sup>4</sup> has announced the discovery of two new antigenic types of *Shigella* with the provisional designations of *Shigella flexneri* type VII and *Sh. flexneri* type VIII. According to Weil types VII and VIII are the same as the Flexners X and Y of Andrewes and Inman<sup>4</sup> which Weil and associates regard as types possessing specific antigens.

The Army sera were made for use in a slide agglutination technique for provisional identification of dysentery organisms, to be followed by determination of motility and confirmatory biochemical studies.

Our experience<sup>10</sup> in this laboratory in the preparation and use of Flexner and Boyd typing sera led us to believe that Wheeler's views on the serology of the Flexner group were better applicable to diagnostic problems than those of Weil and associates.<sup>3</sup> It appeared to us that Wheeler's absorption scheme could be extended and that antisera could be produced which would be free of practically all minor agglutinins. A Flexner antiserum, for example, could be absorbed not only with other cross-reacting heterologous Flexner types for removal of group agglutinins but with any heterologous *Shigella* which gave a reaction in the serum. Such an antiserum should be free of agglutinins for all dysentery organisms except the homologous type. Absorbed antiserum for other species, such as *Shigella dysenteriae*, could be produced by the same method. It was recognized that paracolon and other organisms of the Enterobacteriaceae might possess minor antigens in common with *Shigella*. Only trial of absorbed sera could determine if cross-reactions would occur with bacteria of other genera.

During a period of preliminary trial the typing of dysentery bacteria was attempted with absorbed sera only after biochemical studies had been made. When no disagreements between cultural and serologic tests were noted during the period of trial, it was decided to classify *Shigella* on the basis of serologic reactions alone.

This paper reports the examination of 315 enteric cultures by slide agglutination technique with absorbed *Shigella* sera. By means of this rapid method of examination a report was made on the identity of *Shigella* present in a fecal specimen approximately forty-eight hours after the specimen was received in the laboratory. Eventual fermentation and tube agglutination studies were made on every organism included in the study.

#### METHODS

*Sera.*—The following absorbed sera were provided the Central Diagnostic Laboratory: *Sh. dysenteriae*, *Sh. ambigua*, *Sh. alkalescens* (Type I), *Shigella sonnei*, polyflexner (prepared with V, W, X, Y, Z, 103, P119, and 88 strains), monovalent Flexner V, monovalent Flexner W, monovalent Flexner Z, monovalent Boyd 103, monovalent Boyd P119, and monovalent Boyd 88. Additional absorbed sera were prepared for the mannitol-negative types Sachs Q454, Q771, Q902, Q1030, and Q1167, together with absorbed sera for Boyd types 170, P288, D1, D19, P274, and P143. A polyvalent serum was also prepared to cover Boyd types 170 through P143. The Sachs and Boyd type sera, other than Boyd 103, P119 and 88, were not used routinely.

*Source of Cultures.*—The same stock strains used for production of sera in an earlier study<sup>10</sup> were employed, plus additional cultures.\*

\*Contributed by Dr. Kenneth M. Wheeler, Connecticut State Department of Health, Hartford; Captain C. V. Seastone, Medical Corps, Army Medical Center, Washington, D. C.; Dr. C. A. Peluffo and Dr. Estenilo Hormaeche, Institute of Hygiene, Montevideo, Uruguay; and Dr. Joseph Felsen, The Bronx Hospital, New York City.

*Production of Antisera.*—White rabbits weighing approximately 6 pounds were used for production of antisera. The usual immunization procedure was as follows: A series of three intracutaneous inoculations were made on alternate days with formalinized saline suspensions of cultures grown on blood agar medium. These were followed by six to seven intravenous inoculations of living organisms also grown on blood agar. Intravenous inoculations were made on alternate days.

Suspensions were standardized to the turbidity of a No. 2 McFarland standard, and care was taken not to suspend agar along with organisms. The total amount of inoculum was usually 9.0 cubic centimeters.

Immune sera after processing were preserved with chloroform.

*Preparation of Absorbed Sera.*—Table I on the preparation of a Flexner V antiserum will serve as an example of the steps taken in preparing an absorbed serum. Unabsorbed serum, diluted 1:10, was tested by slide agglutination technique against eighteen-hour, living, smooth strains of *Shigella* grown on blood agar. None of the cultures was heated before use to destroy heat-labile "blocking" antigen, since the cultures listed, including *Sh. alkalescens* R strain, have shown no evidence of possessing such a substance.

The technique of the test was the same as that employed in identification of unknown dysentery cultures by the finished serum. The wells of agglutination slides\* were filled two-thirds full of serum and test organisms were rubbed into the edges of serum drops by means of a loop or straight inoculating wire. An even, milky suspension of organisms resulted which was, admittedly, somewhat uneven in turbidity from well to well. The slide was rocked by hand and observed for fifteen minutes. The system of recording reactions was by plus signs: 4 plus denoted complete agglutination, 2 plus partial agglutination, doubtful a barely detectable clumping. Since speed of reaction was some indication of the potency of a serum, the words "instant," "moderate," and "slow" were used to denote reactions taking place, respectively, as soon as suspension was accomplished, within one minute, or after approximately five minutes.

Following the determination of reactions in unabsorbed serum, strains were selected for removal of cross-reacting agglutinins. With Flexner antisera the X and Y strains were always used for initial absorptions. Organisms for absorption were grown on veal infusion agar in Blake bottles; they were then washed off the agar with saline and spun down in an anglehead centrifuge. The supernatant fluid was poured off and unabsorbed serum, diluted 1:10, was added to the packed bacteria. Resuspension of the bacteria was accomplished by stirring. Incubation was carried out at 50° C. for one hour. An excess of the absorbing dose of bacteria was used for each step in freeing a serum of cross-agglutinins.

After incubation of serum and antigen, bacterial cells were removed by centrifugation, and the serum was tested against each strain which had reacted in the initial titration. In the case of Flexner V antiserum, Lot 3, absorption was completed by a second absorbing dose consisting of Flexner Z and *Sh. alkalescens* organisms. Slide agglutination tests were carried out with Flexner V antiserum and the antigens which had reacted following absorption with X

\*Supplied by Clay-Adams Company, Inc., New York, N. Y.



TABLE I. FLEXNER V ANTISERUM, LOT 3

LIVING ANTIGENS	SLIDE AGGLUTINATION REACTIONS			
	UNABSORBED SERUM (DILUTION 1:10)	FOLLOWING FIRST ABSORPTION* (DILUTION 1:10)	FOLLOWING SECOND ABSORPTION† (DILUTION 1:10)	ABSORBED SERUM (DILUTION 1:20)
Flexner V, 3090	++++	++++ Instant	++++ Instant	++++ Moderate
Flexner V, R	++++	++++ Instant	++++ Instant	++++ Moderate
Flexner VIIa, R	++	-		
Flexner VIIb, C	++	-		
Flexner X694, C	++++	-		
Flexner X1533, C	++++	-		
Flexner X1806, C	++++	-		
Flexner X1807, C	++++	-		
Flexner Y412, A	++++	-		
Flexner Y, R	++++	-		
Flexner Y F1219	++++	-		
Flexner Z, R	++++	++++	-	
Boyd 103, C	++++	++++	-	
Boyd P119, M	++	-		
Boyd P119, A	++	-		
Boyd 88, C	++	-		
Boyd 170, C	++	-		
Boyd P288, M	-	-		
Boyd P288, N.M.	-	-		
Boyd D1, M	-	-		
Boyd D19, M	+	-		
Boyd P274, M	-	-		
Boyd P143, M	±	-		
Lavinton 2525	-	-		
Sachs Q454	-	-		
Sachs Q771	-	-		
Sachs Q902	-	-		
Sachs Q1030	-	-		
Sachs Q1167	-	-		
Sh. dysenteriae 1046	-	-		
Sh. ambigua H	-	-		
Paracolon 339	++	-		
Sh. sonnei	-	-		
Sh. alkalescens R	++++	++++	-	
Salmonella typhi 703	-	-		

++++, Complete agglutination.

++, Agglutination, 50 per cent.

Filter of Flexner V antiserum, absorbed, with homologous strains, 1-2560.

Determination made by tube dilution method, incubation 50° C. for eighteen hours.

\*Absorbing strains: Flexners X694, X1533, X1806, X1807, Y R, Y F1219, and Y412.

†Absorbing strains: Flexner Z and *Sh. alkalescens*.

and Y strains. At this stage the serum appeared free of minor agglutinins, while the reaction with Flexner V organisms was instant and complete. The serum dilution was doubled and tests were again made with Flexner V antigens. When agglutination took place within one minute and was complete, a dilution of 1:20 was selected as the "working dilution."

Only rarely has it been necessary to accept an antiserum diluted 1:10; on the other hand, no serum was diluted more than 1:20 because of possible deterioration. As a preservative, merthiolate in powder form was added to antiserum to make a final concentration of 1:1,000. It was then filtered through a Seitz pad and a final titration made by testing the serum against its homologous strains by tube agglutination technique.

In the example given in Table I, a total of thirty-five strains are listed as antigens. The number has varied from lot to lot of antisera, as new antigenic types have been added to our collection. At present we employ forty-six strains as titration antigens. Storage of these strains dried in vacuo from the frozen state has aided greatly in prevention of dissociation.

The inclusion of *Salmonella typhi* among the stock strains was done originally as an experiment. It was thought a worth-while precaution to remove minor agglutinins for *S. typhi* from Shigella sera since identification of dysentery cultures was to be made from triple sugar slants and since occasional strains of the typhoid organism are found which are dysentery-like in their reactions, failing to produce  $H_2S$  in twenty-four hours. When it was found that strong cross-reactions for *S. typhi* develop in some of the Flexner sera, *S. typhi* 703 was included in all titrations. Whether or not the cross-reactions with *S. typhi* are due to antibodies similar to the alpha agglutinins reported by Stamp and Stone<sup>11</sup> has not been determined.

*Performance of the Diagnostic Slide Agglutination Test.*—The technique of the diagnostic slide agglutination test has varied somewhat with the bacteriologist. In general the method has been as follows: Organisms producing dysentery-like reactions on triple sugar slants were selected for typing. Each culture was tested in trypanflavine solution (1:500 concentration of acriflavine hydrochloride in 0.85 per cent NaCl solution) and in physiologic saline solution for indications of autoagglutinability. It was discovered early in our experience with trypanflavine solution that *Sh. alcalescens* strains possessing heat-labile "blocking" substance were clumped rapidly in trypanflavine,\* yet remained in even suspension in normal saline. Organisms clumping in trypanflavine and not in saline were either (1) placed in saline suspension and boiled for fifteen minutes, then tested with absorbed *Sh. alcalescens* antiserum; or (2) identified by conventional methods, that is, by observation of biochemical activities and reaction in specific serum by tube method. Only strains of *Sh. alcalescens* identified by slide agglutination are included in this report.

Cultures which did not clump in either trypanflavine or saline were tested in polyflexner, *Sh. sonnei*, and *Sh. alcalescens* absorbed sera. Organisms which were agglutinated in either of the last two sera were reported by species. A culture which agglutinated in polyflexner serum was tested in the three monovalent Flexner (V, W, and Z) and three Boyd sera (103, P119, and 88). An organism which reacted in the polyvalent serum and a monovalent serum was reported by species and type, that is, *Sh. paradysenteriae*, Boyd 103. With but few exceptions the reactions in monovalent sera were complete in one or two minutes or less. Flexner W strains were more variable in agglutinability than other types. Occasional strains were clumped rapidly; others, while smooth by test in saline, were slow to react.

*Biochemical Tests.*—All cultures discussed in this report were tested for fermentative reactions in semisolid agar medium containing glucose, lactose, sucrose, maltose, mannitol, and xylose, respectively. Organisms selected for

\*Since some *Sh. dysenteriae*, *Sh. ambigua*, and Sachs strains have been reported as occurring with heat-labile antigens, it is possible that occasional strains will clump in trypanflavine. VI strains of *S. typhi* clump rapidly in trypanflavine.<sup>2-3</sup>

special study, such as strains of Boyd 88 and *Sh. alkalescens*, were inoculated in liquid carbohydrate media. In the case of Boyd 88 cultures, duleitol was added to the list of carbohydrates. An occasional strain of particular interest, such as C-27, together with strains of *Sh. alkalescens*, were tested with a wide range of carbohydrates. Observation of indol production and hydrolysis of urea was done routinely, whereas reactions on Simmons' citrate were observed only on cultures selected for special study. Tests for motility were made on *Sh. alkalescens* cultures and on all strains whose biochemical pattern was at variance with the evidence of tube or slide agglutination.

*Macroscopic Agglutination.*—Tube agglutination tests were carried out at 50° C. in the water bath for eighteen hours on all organisms with dysentery-like reactions in triple and single sugar media. The following antisera were available: *Sh. dysenteriae*, *Sh. ambigua*, *Sh. alkalescens*, *Sh. sonnei*, and polyflexner.

#### RESULTS

A total of 315 cultures which gave dysentery-like reactions in triple sugar agar slants was examined by slide agglutination technique with absorbed sera. Of these, 307 were accepted as *Shigella* on the basis of their agglutination in the spot test. The serologic identification of the 307 cultures was as follows:

<i>Sh. sonnei</i>	159
<i>Sh. paradysenteriae</i>	115
(Flexner and Boyd)	
<i>Sh. alkalescens</i>	32
<i>Sh. ambigua</i>	1
Total	307

The paradysentery types were identified by monovalent sera as:

Flexner V	1
Flexner W	26
Flexner Z	28
Boyd 103	8
Boyd P119	0
Boyd 88	52
Total	115

Five cultures which are included in the total of 315 were agglutinated in polyflexner antiserum on slide test. Four of these failed to react in monovalent sera and were, therefore, not reported as *Shigella*. Fermentation studies revealed that they were aerogenic and belonged to the paracolon group. The fifth culture, 8255, reacted slowly in Flexner W antiserum and was reported as a Flexner W. Subsequent biochemical studies demonstrated that it, also, was a probable paracolon. On first isolation this culture was anaerogenic in triple and single sugars, but it fermented glucose, maltose, and mannitol with production of acid and gas, upon transfer. The organism failed to ferment lactose, sucrose, duleitol, and salicin in twelve days and did not utilize citrate or hydrolyze urea. It was indol positive, nonmotile, and reduced trimethylamine oxide to trimethylamine. Absorption studies with 8255 and Flexner W organisms and their antisera demonstrated that 8255 culture has a minor antigenic

relationship for Flexner W. Agglutinins for 8255 and Flexner W organisms were not present in the serum of the rabbit used for production of 8255 immune serum.

Also included in the total of 315 organisms are two paradysentery cultures which could not be identified by slide agglutination technique. One reacted in polyflexner antiserum but failed to agglutinate in the six monovalent sera. Although it was a typical paradysentery organism biochemically, and smooth in phase, it apparently possessed no type-specific antigen. This strain was classified as Flexner Y on the basis of its 3,4 group antigens. The second paradysentery culture was typical biochemically and unstable in saline suspension upon first isolation. After rapid transfer it was agglutinated in polyflexner antiserum by the tube method. No type determination was made.

Only one of the more exotic *Shigella* was identified in the study of 315 cultures. This organism did not react with the absorbed sera used routinely and was, therefore, put aside for biochemical tests. Of the carbohydrates, glucose, lactose, sucrose, maltose, mannitol, xylose, and dulcitol, it fermented glucose only in twenty-four hours. In ninety-six hours, maltose was fermented weakly. Its other characteristics were those of a *Shigella*. Of the absorbed sera prepared against mannitol-negative *Shigella*, it was agglutinated only in Sachs Q771 serum. An antiserum prepared against our strain 7005 agglutinated a stock strain of Q771 to approximately the titer of the homologous organism. Reciprocal absorption tests demonstrated that 7005 and the stock strain possessed similar antigens.

Since Sachs Q771 strains have been reported infrequently in this country,<sup>15, 16</sup> the source was of interest. Isolation was made from the fecal specimen of a 7-year-old Mexican child hospitalized with dysentery.

The identification of culture 8255 as a Flexner W was a definite error in classification due, unquestionably, to the use of inadequately absorbed W serum. A second error, also brought about by reliance on serologic identification alone, must be viewed in a different light. Among the 159 organisms classified as *Sh. sonnei* was a culture which gave atypical reactions biochemically. This culture, C-27, was found to be motile and anaerogenic, fermenting glucose, lactose, and maltose of the commonly used carbohydrates. By absorption studies it was found to possess the major antigen of *Sh. sonnei*, phase I, as defined by Wheeler and Mickle.<sup>17</sup> A description of C-27 will be made in detail in a separate publication since this organism poses an interesting problem in classification.

#### *Biochemical Tests.*—

*Flexner Types:* All of the sixty-five Flexner organisms typed with absorbed sera were typical in their fermentation reactions, fermenting glucose and mannitol, or glucose, maltose, and mannitol. None fermented xylose. The majority produced indol.

*Boyd 103:* Boyd 103 types were irregular in their reactions. Six strains from the same source fermented glucose only of the carbohydrates used routinely. Mannitol was not fermented after prolonged incubation; indol was formed by

all six strains. Antisera were produced against two of the cultures and absorption studies were carried out with stock antisera and stock cultures of Boyd 103. The mannitol-negative strains were able to exhaust the agglutinins of a Boyd 103 stock antiserum for both stock cultures and mannitol-negative strains. The same results were obtained when sera for the mannitol-negative Boyd 103 types were absorbed with stock cultures.

Two of the eight Boyd 103 types identified were typical in their reactions, fermenting glucose and mannitol in twenty-four hours. One strain produced indol and the other did not.

*Boyd 88:* All but three of the fifty-two Boyd 88 types classified serologically were from a single outbreak, and, as might be expected, the forty-nine strains from the outbreak were fairly similar in biochemical characteristics. All fermented glucose and mannitol promptly; the majority fermented maltose in six or seven days, whereas dulcitol was attacked slowly by most strains and a few did not ferment the carbohydrate in fourteen days.

None of the fifty-two strains produced indol. One strain, not from the outbreak, fermented glucose and mannitol only. The irregularity of fermentative reactions by organisms of the Boyd 88-Newcastle-Manchester series has been noted by numerous investigators<sup>5, 18, 19</sup> and has been attributed as regards gas production to the brand of peptone used as a base for fermentation media.<sup>20</sup> Two non-motile, gas-forming cultures were examined by us which were identified serologically as type Boyd 88. Both appeared anaerogenic on triple sugar medium in twenty-four hours but produced a small amount of gas in forty-eight hours. Both fermented glucose and mannitol promptly, eventually producing a considerable bubble of gas in the Durham vials. Neither culture fermented dulcitol in eight days. Absorption studies demonstrated that both organisms possessed the major antigen of type Boyd 88.

*Shigella sonnei:* With the exception of culture C-27, the organisms identified as *Sh. sonnei* were typical in their reactions during a short period of incubation. All fermented glucose, maltose, and mannitol in twenty-four hours; none fermented xylose or produced indol. Prolonged incubation of the Sonne cultures was not made to obtain reactions in lactose and sucrose.

*Shigella alkalescens:* Stuart and associates<sup>21</sup> have pointed out that the antigenic and biochemical limits to the species *Sh. alkalescens* are difficult to fix. They report a study of 141 cultures corresponding to the classical description of *Sh. alkalescens* and forty-two cultures intergrading biochemically from *Sh. alkalescens* to *Escherichia coli* which possessed some or all of the antigenic fractions of *Sh. alkalescens*. It would appear that the identification of this species according to the strict definition is impossible by serologic means alone.

In order to discover what had been labeled as *Sh. alkalescens* by the slide agglutination test, strains isolated by us were examined in a wide range of carbohydrates and were grown on Simmons' citrate, urea medium, Voges-Proskauer broth, and semisolid motility agar. The majority of the cultures were typical in reaction, fermenting glucose, maltose, mannitol, and dulcitol, forming indol, and failing to grow on citrate or to produce acetyl-methyl-carbinol. They

were nonmotile. There were ten exceptions which were atypical in reaction with the difference that they grew on citrate in three to five days and were motile.

*Shigella ambigua*: The one *Sh. ambigua* culture identified serologically was typical in its biochemical reactions and was nonmotile.

*Tube Agglutination*: The 307 organisms classified as *Shigella* by slide agglutination technique were agglutinated to complete or moderate titer with appropriate antisera, with but few exceptions. An occasional strain was found to be partially dissociated by the time the tube method was employed and required transfer before a repeat test. Boyd 88 strains were not agglutinated strongly in the polyflexner serum employed for the tube agglutination titer, the average end point being 1:320. In contrast, Flexner strains were agglutinated in a dilution of 1:1280 to 1:5120 in the same serum. The average end point for *Sh. sonnei* strains in specific serum was a dilution of 1:5120. All of the *Sh. alkalescens* strains were agglutinated strongly in unabsorbed specific serum.

#### DISCUSSION

The rapid identification of *Shigella* by the use of absorbed sera has become an established procedure in some laboratories, since there are definite advantages in its employment in the slide technique over the tube method with unabsorbed sera. It is now a question with those who have adopted the slide agglutination technique of how far shall we go in accepting the results of serologic classification. Shall we report the identity of an organism as *Shigella* solely on the results of serologic identification with highly absorbed sera, or shall we combine serologic identification with biochemical confirmation studies to arrive at the identity of a dysentery-like organism?

The first course is attractive because of the rapidity with which a report may be made to a physician. Instead of a period of seventy-two to ninety-six hours between receipt of a specimen and a report on the *Shigella* present, there is the possibility of a report in forty-eight, or even twenty-four, hours if typing is made direct from an isolation plate. To offset the advantage of speed there is the possibility of an error in identification.

The chances of error are not great but they are definite. There is first the possibility that all cross-reacting agglutinins have not been absorbed from sera. An error due to inadequate absorption of Flexner W antiserum is reported in the present study. There is the possibility of reaction between absorbed serum and its specific antigen existing in a different species, as in the case of culture C-27, resulting, nevertheless, in an incorrect identification.

Just what the significance of an organism such as C-27 is in the scheme of the Enterobacteriaceae is uncertain; one would hesitate to classify this culture as *Sh. sonnei* from our present imperfect knowledge of the species and of organisms related to it antigenically. Wheeler and Stuart<sup>16</sup> have described a motile paracolon strain with the complete somatic antigen of Sachs type Q771, and it seems possible that similar relationships between paracolon and *Shigella* organisms will be discovered in the future. Whether these related but atypical

organisms will be numerous enough to cause frequent errors in classification seems very doubtful. The paucity of reports in the literature would indicate that such cultures are few.

Should classification in the present study of ten motile, citrate-positive strains as *Sh. alkalescens* be considered as further error due to serologic methods? According to the present limits of the species, yes. It is evident, however, from the work of Stuart and co-workers<sup>21</sup> that a new conception of the species may be realized and that classification according to the present limits may be changed.

Since *Sh. alkalescens* isolated from feces is not reported to the physician by our laboratory, any discussion of error in the classification of the motile, citrate-positive organisms is academic. It seems probable that many of these cultures have been, and are being, classified as *Sh. alkalescens* on the basis of conventional methods of identification. Motility is usually sluggish and is noticeable in semisolid medium only after twenty-four to thirty-six hours' incubation, while the reaction on citrate is slow, alkalinity appearing in three to five days.

In view of the results in the present study, it would appear that the chances of error in identifying the pathogenic *Shigella* by use of highly absorbed sera are slight. If we except from discussion the controversial issue of the limits to the species *Sh. alkalescens*, our rate of error was two out of 284 specimens, or approximately 0.7 per cent. When one considers methods of identification of several years ago, the results by the rapid method look very good indeed. How then would the mannitol-negative Boyd 103 strains have been classified? Would a report on either the Boyd 103 or the aerogenic Boyd 88 types have been made within a reasonable period of time in order that they might be of use to a physician?

A contrast of past and present methods in which present methods appear to advantage should not overlook the fact that the latter are subject to occasional errors. Therefore, at the completion of this study, our recommendation was as follows: A provisional report on the identity of *Shigella*-like organisms should be made, based on the outcome of the slide agglutination test. In our opinion the value of a rapid report to the physician justifies this procedure. Confirmatory motility and biochemical studies should continue to be made on each strain examined. This should be done both for confirmation of serologic results and to add to our knowledge of irregular biochemical reactions among the *Shigella*.

#### SUMMARY

1. Methods for production of highly absorbed *Shigella* antisera for use in the slide agglutination test are presented.

2. A total of 315 cultures which appeared dysentery-like on triple sugar agar was examined by slide agglutination technique with absorbed sera. Of these, 307 were accepted as *Shigella* on the basis of their reactions in absorbed sera. One of the 307, identified as *Sh. sonnei*, was found to be atypical in fermentation reactions and motile, yet it possessed the major antigen of *Sh. sonnei*.

Of thirty-two cultures identified serologically as *Sh. alkalescens*, ten were atypical in that they utilized citrate slowly and were motile. The remainder the 307 cultures were confirmed as *Shigella* by means of biochemical and tube agglutination tests.

3. One Sachs type Q771 strain was identified.

4. Rapid identification of two paradysentery strains could not be made because of "rough" phase in one culture and lack of specific antigen in the other.

5. Of five paracolon strains which reacted with polyvalent Flexner antiserum, four were eliminated as pathogens because of failure to react with monovalent serum; the fifth was incorrectly identified as Flexner W.

6. It was concluded that immediate reports on the identity of *Shigella* are justified when they are based on the rapid slide agglutination test, provided antisera used in the test have been highly absorbed. All identifications should be followed by tests of motility and biochemical studies.

#### REFERENCES

1. Wheeler, K. M.: Serological Identification of Dysentery Bacilli, *Am. J. Pub. Health* 34: 621, 1944.
2. Wheeler, K. M.: Antigenic Relationships of *Shigella* Paradysenteriae, *J. Immunol.* 48: 87, 1944.
3. Weil, A. J., Black, J., and Farsetta, K.: The Serological Types of *Shigella* Paradysenteriae (Flexner), *J. Immunol.* 49: 321, 1944.
4. Andrewes, F. W., and Inman, A. C.: A Study of the Serological Races of the Flexner Group of Dysentery Bacilli, *Med. Res. Committee, Special Report Series No. 42*, London, 1919.
5. Boyd, J. S. K.: The Antigenic Structure of the Mannitol-Fermenting Group of Dysentery Bacilli, *J. Hyg.* 38: 477, 1938.
6. Francis, A. E.: Two New Types of *Shigella* Flexneri, *J. Path. & Bact.* 58: 320, 1946.
7. Weil, A. J., Barnes, L. A., and Binder, M.: Immunological Relations Between Types of *Shigella* Paradysenteriae (Flexner) and Other *Shigellae*, *J. Immunol.* 51: 133, 1945.
8. Nelson, J. B., and Tenbroeck, C., and Dammin, G. J.: Distribution of *Shigella* in India as Determined by Spot Agglutination With Absorbed Sera, *Am. J. Pub. Health* 35: 1282, 1945.
9. Research Section, Division of Bacteriology, Army Medical School: Typing Sera for the Genus *Shigella*, *Bull. U. S. Army M. Dept.* 4: 627, 1945.
10. Ferguson, W., and Carlson, M.: Rapid Identification of *Shigella* by Serologic Methods, *J. Bact.*, Abstract 49: 526, 1945.
11. Stamp, L., and Stone, D. M.: An Agglutinin Common to Certain Strains of Lactose and Non-Lactose-Fermenting Coliform Bacilli, *J. Hyg.* 43: 266, 1944.
12. Schutze, Harry: Agglutinability of *Bact. Shigae*, *J. Path. & Bact.* 56: 250, 1944.
13. Weil, A. J., Binder, M., and Slafkovsky, H.: *Shigella* Sp. Wakefield and Its Relations to *Shigella* Sp. Sachs Q454, *J. Immunol.* 53: 119, 1946.
14. Hormaeche, E., and Peluffo, C. A.: Salmonellosis in Infancy and Its Diagnosis, *Puerto Rico J. Pub. Health & Trop. Med.* 17: 99, 1941.
15. Gober, M., Stacy, V., and Woodrow, M.: A Probably New Type of Nonmannitol-Fermenting *Shigella*, *Am. J. Hyg.* 40: 209, 1944.
16. Wheeler, K. M., and Stuart, C. A.: The Mannitol-Negative *Shigella* Group, *J. Bact.* 51: 317, 1946.
17. Wheeler, K. M., and Mickle, F. L.: Antigens of *Shigella Sonnei*, *J. Immunol.* 51: 257, 1945.
18. Downie, A. W., Wade, E., and Young, J. A.: An Organism Resembling the Newcastle Type of Dysentery Bacillus Associated With Cases of Dysentery, *J. Hyg.* 33: 196, 1933.
19. Clayton, P. H. A., and Warren, S. H.: An Unusual Bacillus Recovered From Cases Presenting Symptoms of Dysentery, *J. Hyg.* 28: 335, 1929.
20. Ewing, J. O., and Taylor, J.: Variations in the Fermentative Reactions of Antigenically Identical Strains of *Bact. Newcastle*, *Month. Bull. Min. Health & Emerg. Pub. Health Lab. Serv.* 4: 130, 1945; abstracted in *Bull. Hyg.* 20: 576, 1945.
21. Stuart, C. A., Rustigan, R., Zimmerman, A., and Corrigan, P. W.: Pathogenicity, Antigenic Relationships, and Evolutionary Trends of *Shigella Alkalescens*, *J. Bact.* 47: 425, 1943.



# THE SEROLOGIC RESPONSE IN MURINE TYPHUS AS MEASURED BY THE WEIL-FELIX, RICKETTSIAL COMPLEMENT FIXATION, AND RICKETTSIAL AGGLUTINATION REACTIONS

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SINCE its introduction in 1916, the Weil-Felix test<sup>1</sup> has been a valuable aid in the diagnosis of rickettsial diseases in human beings. This reaction, based on the development of nonspecific agglutinins for the O or nonmotile variants of certain strains of *Proteus* X, has been widely used for diagnostic purposes because of its simplicity and accuracy in detecting rickettsial infections. In common with other nonspecific serologic reactions, however, the Weil-Felix test has limitations which, if not restricting its usefulness, often render its interpretation difficult. For example, it is now generally agreed that the test does not distinguish between typhus and the rickettsial diseases of the spotted fever group. This is of some importance in regions such as the Southern United States where murine typhus and Rocky Mountain spotted fever are both endemic. Moreover, the sera of normal persons who have never been exposed to rickettsial infection often contain low-titer agglutinins for *Proteus* X strains. Consequently it is usually not safe to draw diagnostic conclusions from the results of a single Weil-Felix test, even if the titer exceeds a level commonly regarded as significant. Another source of difficulty in assessing the significance of the Weil-Felix reaction may arise from the fact that agglutinins for *Proteus* X strains sometimes occur as a result of infection with *Proteus vulgaris* and *Proteus mirabilis*.<sup>2</sup>

With the development of improved methods for cultivating rickettsiae in relatively large amounts, it has become feasible to employ purified rickettsial suspensions as antigens in the serologic diagnosis of typhus and related diseases. When such antigens are used in complement fixation or agglutination tests they afford convenient means for diagnosing rickettsial infections. In addition, their greater specificity permits a differentiation between the various rickettsial diseases which give overlapping results with the Weil-Felix test.<sup>3-14</sup> The antigens most commonly used at the present time are prepared by growing rickettsiae in the yolk sac of the developing chick<sup>15</sup> or in the lungs of rodents.<sup>16, 17</sup>

It is evident from data published thus far that there has been little occasion for studying the comparative effectiveness of suspensions of rickettsiae and *Proteus* OX strains in the early serologic diagnosis of murine typhus. As Felix has pointed out,<sup>18</sup> murine typhus is essentially a sporadic disease with a relatively low fatality rate, and patients usually do not come under observation soon enough to permit an adequate investigation of the pattern of serologic

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response in the early stages of infection. The studies reported here were undertaken primarily to investigate the course of antibody development in patients with murine typhus by comparing the results of the Weil-Felix test with the specific rickettsial agglutination and complement fixation reactions. It was hoped that in this way it might be possible to evaluate more clearly the relative usefulness of the murine rickettsial agglutination and complement fixation tests in the early diagnosis of the disease.

#### METHODS AND MATERIALS

For the purposes of this study, multiple samples of blood serum were collected over a period of several weeks from patients with clinical evidence of typhus infection. In almost all instances these patients were residents of communities in the Southeastern United States where murine typhus is endemic. Efforts were made to obtain samples of serum at intervals of two to four days during the first three weeks of illness and at seven or eight days for the next five weeks. Thereafter, whenever possible, serum was collected once each month until a period of 150 days following the onset of illness had elapsed. In many cases, however, the patient could not be observed beyond the initial eight-week period. Although it was thus possible to obtain accurate data on the development of antibody levels, the persistence of titers in these cases could only be partially studied.

Sera collected in this manner were sent to a central laboratory where agglutination and complement fixation reactions with murine rickettsial antigens\* as well as agglutination tests for Proteus strains OX 19, OX 2, and OX K were performed with each specimen. Sometimes complement fixation tests were also carried out with Rocky Mountain spotted fever rickettsial antigen. The serum specimens were tested as they were received at the central laboratory. In addition, the unused portions of successive sera from any one patient were stored at 4° C. and later retested simultaneously by each method. This was done to safeguard against possible variations in the reactivity of the antigenic suspensions. Only those patients who gave an accurate history regarding date of onset of symptoms and in whom it was possible to trace the progressive development of antibody titers during illness have been included in this report. Of the thirty-four nonfatal cases of murine typhus originally comprising this series, twenty-two could be studied sufficiently closely to yield accurate and comparable data.

The Proteus OX antigens employed in the Weil-Felix tests were alcohol-treated suspensions prepared and standardized according to Bridges' modification of Bien's method.<sup>19, 20</sup> Prior to use, the concentrated suspensions were adjusted to a density approximating the McFarland nephelometer standard No. 3. For the agglutination tests, 0.5 c.c. amounts of suspension were added to 100 by 13 mm. round-bottomed tubes containing equal volumes of the patient's serum which had been made up in serial twofold dilutions ranging from 1:10 to 1:640. All tests were controlled with positive and negative sera. The tubes were centrifuged at 2,000 r.p.m. for seven minutes and then read macroscopically after shaking slightly to dislodge the particles from the bottom of each tube. The highest serum dilution showing any degree of agglutination was recorded as the titer. The reaction was arbitrarily considered to be positive when agglutination occurred in an initial serum dilution of 1:80 or more or when a fourfold increase in titer over a previous specimen had been observed.

Rickettsial agglutination tests were performed with antigens obtained from infected duck sacs of developing chick embryos.<sup>13</sup> The rickettsial suspensions were prepared in the manner recommended by Fitzpatrick,<sup>14</sup> using the ether-extraction method of purification. Such suspensions were found to be relatively stable, retaining their original specificity and reactivity for a period of at least six months when stored at refrigerator temperatures. Serum specimens for examination were diluted serially from 1:20 to 1:160 and tested by the slide agglutination technique previously described.<sup>14</sup> Positive and negative control sera were

\*The murine rickettsial antigens for agglutination tests were provided by the courtesy of Florence K. Pitts, A. Dohme, Inc., Glenolden, Pa. The rickettsial antigens used in the complement fixation studies were supplied through the kindness of Dr. Ida A. S. S. Nation, Bethesda, Md.

included in each test. The slides were placed in the incubator at 40° C. for five hours and then stored at 4° C. overnight. Preliminary macroscopic readings were made after the slides had remained in the incubator for one and five hours, respectively. A final reading was made with the low-power objective of the microscope after overnight refrigeration. The test was recorded as positive only when complete agglutination was observed in an initial serum dilution of 1:40 or more. When agglutination occurred it usually was visible to the unaided eye within a period of one hour. In reading results the slide test presented none of the difficulties encountered by van Rooyen and associates<sup>12</sup> with rickettsial agglutination reactions in test tubes.

The antigens used for the complement fixation tests were also prepared from rickettsiae grown in the yolk sac of embryonated hen eggs.<sup>15</sup> The rickettsial suspensions were made up and standardized by the method employed by Bengtson.<sup>21</sup> Serum samples from each patient were inactivated at 56° C. for one-half hour and then diluted serially from 1:4 to 1:512. The technique of the complement fixation test was that described by Bengtson,<sup>21</sup> using the customary reagent controls. Each day's tests were controlled also with positive and negative sera. The degrees of fixation were recorded as 4, 3, 2, and 1 plus, and trace. In this test a reaction of 3 or 4 plus in a serum dilution of 1:8 or higher was considered to be positive.

### RESULTS

The results of the serologic studies in the twenty-two patients with murine typhus included in this series are summarized in Table I. Generally speaking, in most of these patients the Weil-Felix and rickettsial agglutination tests became positive earlier in the course of illness than did the rickettsial complement fixation reaction. Sometimes the Weil-Felix became positive slightly sooner than the rickettsial agglutination test; occasionally the reverse was true. For the most part, however, these reactions differed very little in this respect. In only one case was the rickettsial complement fixation test found positive before the other two.

TABLE I. EARLY SEROLOGIC RESPONSE, BY DAY OF DISEASE, IN TWENTY-TWO PATIENTS WITH MURINE TYPHUS

DAY OF DISEASE	NUMBER OF PATIENTS SHOWING A POSITIVE TITER BY		
	WEIL-FELIX	MURINE RICKETTSIAL AGGLUTINATION	MURINE RICKETTSIAL COMPLEMENT FIXATION
4	0	0	0
6	1	1	1
8	2	2	2
10	9	9	6
12	16	15	7
14	20	19	9
16	21	19	12
18	21	20	13
20	22	20	14
22	22	22	15

During the first week of illness all three tests seemed to be equally ineffective in establishing a serologic diagnosis of murine typhus. In the second week of illness the Weil-Felix and rickettsial agglutination tests were usually positive and were clearly superior to the complement fixation reaction in detecting the disease. Even three weeks after onset, when the two agglutination tests were positive in all instances, the complement fixation reaction was still negative in nearly one-third of the cases. In one patient the presence of complement-fixing

antibody was not demonstrated until six weeks after illness began. Further data, comparing the per cent of positive Weil-Felix, rickettsial agglutination, and rickettsial complement fixation reactors by day of disease, are shown in Fig. 1.

These findings suggest that the Weil-Felix and rickettsial agglutination tests are more valuable than the rickettsial complement fixation reaction in the early serologic diagnosis of murine typhus. The rickettsial agglutination reaction, in turn, is superior to the Weil-Felix test in that it is more specific and permits a differentiation to be made between the murine and spotted fever types of infection. In view of this one may conclude that the rickettsial slide agglutination test, as performed in this work, is a superior serologic method for detecting murine typhus in the early stages of the disease.

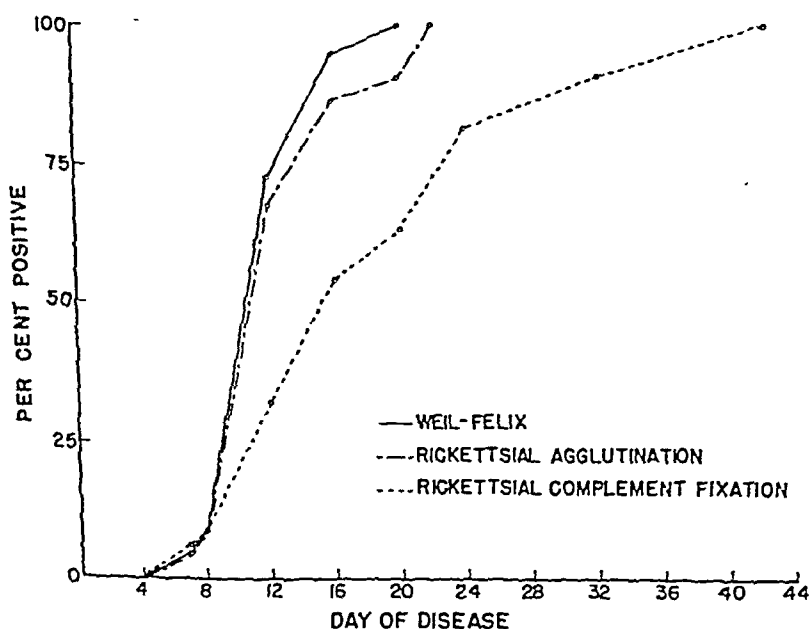


FIG. 1.—Per cent of positive Weil-Felix, rickettsial agglutination, and rickettsial complement fixation reactors, by day of disease, in twenty-two cases of murine typhus.

Additional information gained in the course of these studies indicates that once the rickettsial complement fixation reaction becomes positive it remains so for a relatively long period of time. In this respect it does not appear to differ significantly from the rickettsial agglutination test. Although the persistence of antibody levels in these patients could not be studied completely, it seems clear that the Weil-Felix is the most evanescent of the three reactions and frequently becomes negative eight to twelve weeks after the onset of symptoms.

#### DISCUSSION

According to Felix,<sup>18, 22</sup> the majority of cases of louse-borne typhus and many cases of murine typhus can be diagnosed by the OX 19 reaction during the first week of illness. This has been denied by other investigators<sup>12, 23</sup> who

point out that significant agglutinin titers for rickettsiae and *Proteus* OX 19 usually are not attained by such patients until early in the second week of disease. In so far as murine typhus is concerned, the data presented here indicate that in most cases the disease cannot be diagnosed by serologic methods until after the eighth day of illness.

From time to time in the past, certain workers have postulated that the pattern of agglutinin formation in cases of typhus is related to the clinical course of the disease.<sup>18, 24</sup> Generally speaking, patients with cases of moderate severity are said to respond with high titers, while those with most severe cases, including those who succumb to the infection, have very low titers. In this small series of patients, little or no relationship between the severity of illness and the magnitude of antibody response was found. While this work was in progress it was possible to observe three patients with uncomplicated murine typhus which terminated fatally. In each of these the agglutinin and complement-fixing titers reached high levels prior to death. Although the number of patients studied does not permit one to draw definite conclusions, it seems that the antibody levels attained in murine typhus are subject to wide individual variations and that if an inverse relationship exists between clinical severity and the height of antibody response it must be a tenuous one.

The specificity of rickettsial agglutination and complement fixation reactions already has been demonstrated conclusively by several investigators.<sup>4, 8, 10, 12-14, 17, 25</sup> In the absence of previous rickettsial infection or vaccination, sera from normal persons and individuals suffering from nonrelated febrile illnesses almost never contain antibodies for rickettsial antigens. This was amply confirmed during the course of these studies. On the other hand, some degree of cross agglutination and complement fixation with murine, classic, and spotted fever antigens may be expected to occur in sera from patients with typhus and spotted fever. When the rickettsial suspensions are properly standardized, however, the titer for the infecting strain is usually significantly higher than the heterologous antibody levels. Thus a serologic differentiation may be made between diseases of the typhus and spotted fever groups by means of the agglutination and complement fixation tests.

Plotz and associates<sup>26</sup> have recently indicated that the rickettsial agglutination reaction is less effective than the complement fixation test in differentiating between typhus and Rocky Mountain spotted fever. These investigators, using an unspecified macroscopic agglutination technique, found that the sera from thirteen patients with Rocky Mountain spotted fever gave considerable agglutination with murine and classic rickettsiae, whereas no cross reactions for the typhus antigens occurred in the rickettsial complement fixation test. During the present study there was an opportunity to observe the serologic response in three patients with Rocky Mountain spotted fever. In two of these no cross agglutination with murine typhus antigen was noted in the slide agglutination test. In the third patient partial agglutination in low titer (1:40) occurred with murine rickettsiae, but this titer remained stationary throughout the disease. Sera from all three patients, however, gave slight to moderate cross reactions with murine

rickettsial suspensions in the complement fixation test. While it is obviously impossible to generalize from scattered observations of this sort, such findings tend to reaffirm the value of the slide agglutination test used in these studies for the differentiation of rickettsial diseases. Moreover, from a clinical standpoint, these results emphasize that the more elaborate complement fixation reaction probably possesses no differential diagnostic virtues which are not also shared by the simple slide agglutination test.

# SUMMARY

1. An investigation of the serologic response in twenty-two cases of murine typhus has shown that the Weil-Felix and rickettsial agglutination reactions usually become positive earlier than the rickettsial complement fixation test in this disease.

2. Because of its specificity and sensitivity, the rickettsial slide agglutination test employed in these studies is particularly useful in the early serologic diagnosis of murine typhus.

3. In so far as the detection of murine typhus in its acute stages in man is concerned, the relatively simple rickettsial slide agglutination test is fully as effective as the more elaborate complement fixation reaction.

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# REFERENCES

1. Weil, E., and Felix, A.: Das serologische Diagnose des Fleckfiebers, *Wien. klin. Wchnschr.* 29: 33, 1916.
2. Dammin, G. J., and Billings, F. T.: The Weil-Felix Reaction in Patients With *Proteus* and *Pseudomonas Aeruginosa* Infections, *J. Immunol.* 44: 251, 1942.
3. Bengtson, I. A.: Complement Fixation in "Q" Fever, *Proc. Soc. Exper. Biol. & Med.* 46: 665, 1941.
4. Bengtson, I. A.: Complement Fixation in Endemic Typhus, *Pub. Health Rep.* 56: 649, 1941.
5. Bengtson, I. A., and Topping, N. H.: The Specificity of the Complement Fixation Test in Endemic Typhus Fever Using a Rickettsial Antigen, *Pub. Health Rep.* 56: 1723, 1941.
6. Bengtson, I. A., and Topping, N. H.: Complement Fixation in Rickettsial Diseases, *Am. J. Pub. Health* 32: 48, 1942.
7. Plotz, H., and Wertman, K.: The Use of the Complement Fixation Test in Rocky Mountain Spotted Fever, *Science* 93: 441, 1942.
8. Plotz, H.: Complement Fixation Test in Rickettsial Diseases, *Science* 97: 20, 1943.
9. van Rooyen, C. E., and Bearcroft, W. G. C.: Rickettsial Agglutination Tests in Middle East Forces and Egypt, *Edinburgh M. J.* 50: 257, 1943.
10. Stuart-Harris, C. H., Reittie, G. K. C., and Oliver, J. O.: Rickettsial Agglutination Studies in Typhus Fever, *Lancet* 2: 537, 1943.
11. Kligler, I. J., and Olejnik, E.: Specific Diagnosis of Typhus Fever by Rickettsial Agglutination, *Nature* 152: 627, 1943.
12. van Rooyen, C. E., Bowie, J. H., and Krikorian, K. S.: Typhus Research in Egypt, Palestine, Iraq, and Iran, *Tr. Roy. Soc. Trop. Med. & Hyg.* 38: 133, 1944.
13. Castaneda, M. R.: Differentiation of Typhus Strains by Slide-Agglutination Tests, *J. Immunol.* 50: 179, 1945.
14. Fitzpatrick, F. K.: Studies on Rickettsial Agglutination in Typhus, *J. LAB. & CLIN. MED.* 30: 577, 1945.
15. Cox, H. R.: Use of Yolk Sac of Developing Chick Embryo as Medium for Growing Rickettsiae of Rocky Mountain Spotted Fever and Typhus Groups, *Pub. Health Rep.* 53: 2241, 1938.
16. Castaneda, M. R.: Experimental Pneumonia Produced by Typhus Rickettsiae, *Am. J. Path.* 15: 467, 1939.

17. Hudson, N. P.: A Macroscopic Agglutination Test With Typhus Rickettsiae Prepared From Infected Rodent Lungs, *J. Infect. Dis.* 67: 227, 1940.
18. Felix, A.: Technique and Interpretation of the Weil-Felix Test in Typhus Fever, *Tr. Roy. Soc. Trop. Med. & Hyg.* 37: 321, 1944.
19. Bridges, R. F.: Preparation of Suspensions for Weil-Felix Test, *J. Roy. Army M. Corps* 64: 153, 1935.
20. Bridges, R. F.: Note on the Preparation of Suspensions for the Weil-Felix Test, *Tr. Roy. Soc. Trop. Med. & Hyg.* 37: 343, 1944.
21. Bengtson, I. A.: Complement Fixation in the Rickettsial Diseases—Technique of the Test, *Pub. Health Rep.* 59: 402, 1944.
22. Felix, A.: Discussion in Findlay, G. M.: Laboratory Investigations on Typhus, *Proc. Roy. Soc. Med.* 35: 160, 1941.
23. van den Ende, M., Harries, E. H. R., Stuart-Harris, C. H., Steigman, A. J., and Cruickshank, R.: Laboratory Infection With Murine Typhus, *Lancet* 1: 328, 1943.
24. Reitler, R., Btsh, S., and Marberg, K.: Endemic Typhus in Palestine, *Tr. Roy. Soc. Trop. Med. & Hyg.* 33: 197, 1939.
25. Bengtson, I. A.: Applications of the Complement Fixation Test in the Study of Rickettsial Diseases, *Am. J. Pub. Health* 35: 701, 1945.
26. Plotz, H., Bennett, B., Wertman, K., and Snyder, M.: Cross Reacting Typhus Antibodies in Rocky Mountain Spotted Fever, *Proc. Soc. Exper. Biol. & Med.* 57: 336, 1944.

# INFLUENZA B IN NEEDHAM, MASSACHUSETTS, DECEMBER, 1945

## SEROLOGIC STUDIES IN CASES AND IN FAMILY CONTACTS

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ATTENTION was first called to the possibility of a sizable outbreak of influenza in Needham, Mass., by an abrupt rise in the number of absentees in the Junior High School during the second week of December, 1945. Through the cooperation of the local school and health authorities and of many of the residents of the community, an opportunity was afforded to study certain aspects of this outbreak. The present paper includes a report of serologic studies made in a limited number of cases and family contacts. The epidemiologic observations are reported elsewhere.<sup>1</sup>

### MATERIALS AND METHODS

*Collection of Materials.*—Visits were made to the Junior High School and to the homes of a number of pupils who were acutely ill with influenza between Dec. 13 and 16. Throat washings were obtained at this time from several individuals who were still acutely ill with the symptoms of influenza and with a fever of 101° F. or higher. At the same time samples of venous blood for serologic studies were obtained aseptically from individuals with acute influenza and from members of their families, including both persons with influenza and apparently healthy contacts. Clinical histories were also obtained at this time. A second visit was made to each of the same families between Dec. 29, 1945, and Jan. 2, 1946. At that time blood was again drawn from patients and contacts and a history was obtained of any illnesses that may have occurred during the intervening period. As far as could be ascertained, none of the patients or contacts had received inoculations of influenza vaccines.

*Clinical Findings.*—The clinical features of the cases of influenza were quite characteristic and consisted of fever (100 to 104° F.), prostration, eyeball pain, generalized aching, some cough, and occasionally a mild sore throat. There was usually slight conjunctival injection and supraorbital edema. A few scattered râles were heard in the lungs of some of those individuals who were examined. The severity of the illness was rated as 1 to 3 plus, according to the degree of prostration, height and duration of fever, and the severity of the systemic symptoms. Four adult contacts of patients with influenza had slight fever and predominantly gastrointestinal symptoms which had been called "in-

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testinal flu." There were four other contacts, one a cafeteria worker at the school and three parents of individuals with influenza, who were afebrile and had symptoms characteristic of a common cold, with coryza predominating.

*Antigens.*—The antigens used in the serologic tests included the PR8 strain of influenza A, the Lee strain of influenza B, strains WC and MF isolated from throat washings obtained on Dec. 14 from patients with acute influenza among the Thorndike Laboratory staff who had no contact with the Needham cases, and strain MB isolated from throat washings on the first day of illness from one of the patients in the Needham outbreak (patient 6 in Table I). The PR8 and Lee viruses were originally obtained from Dr. Thomas Francis, Jr., and the others were isolated by amniotic inoculation and passage and were subsequently transferred through more than twenty allantoic passages. Each was concentrated from a pooled harvest of allantoic fluid by elution from the embryonic erythrocytes according to the method of Francis and Salk<sup>2</sup> and kept in a CO<sub>2</sub> icebox at about -70° Centigrade. Details of the isolations of influenza strains during this epidemic will be presented elsewhere.

*Serologic Tests.*—The sera were kept in an electric refrigerator (deep freeze) at -20° C. in rubber stoppered Pyrex tubes and rapidly thawed each time before any was withdrawn for the tests. All serologic tests were carried out in two-fold dilutions of serum and saline beginning with 1:4. Paired sera from each person always were tested at the same time, usually with two or more viruses. A majority of the tests were repeated on two or more separate occasions. At least on one occasion in most cases, dilutions of the sera made up at one time were run simultaneously with three or four of the viruses in both agglutinin inhibition and complement fixation tests. The agglutinin inhibition tests were set up essentially as described by Hirst.<sup>3</sup> Chicken blood for these tests was obtained from a local slaughterhouse and the blood from three or more chickens was pooled each time. The details of the complement fixation test were similar to those described by Enders and associates<sup>4</sup> for the mumps virus. Titers were recorded as the reciprocal of the greatest initial dilution of serum before adding other reagents. The end point in the Hirst test was read at the dilution in which erythrocyte agglutination was completely absent (0) or estimated up to 25 per cent (+). In the latter event the titer was arbitrarily reduced by one-half of the value of the preceding interval. (Example: Complete inhibition in 1:64 and plus agglutination in 1:128; titer is 128 less 32 or 96.) In the complement fixation test the end point was graded 1 to 4 plus, the former representing an estimated 75 per cent hemolysis and the latter complete fixation, that is, no hemolysis. The 1, 2, and 3 plus readings were reduced  $\frac{3}{4}$ ,  $\frac{1}{2}$ , and  $\frac{1}{4}$ , respectively, of the value of the preceding interval. Examples: No hemolysis (4 plus) in 1:64 and complete hemolysis (0) in 1:128; titer is 64. No hemolysis (4 plus) in 1:64 estimated 25 per cent hemolysis (3 plus) in 1:128, and complete hemolysis (0) in 1:256; titer is 128 less  $\frac{1}{4}$  of 64 or 112.) The titers of serum which showed no antibody in the initial dilution (1:4) were arbitrarily given the value of 2.

## RESULTS

*Virus Isolations.*—Attempts to isolate a virus from the throat washings obtained from patients with acute clinical influenza in the Needham outbreak were unsuccessful in most instances. The washings had been kept chilled during transit and stored in sealed Pyrex tubes in a CO<sub>2</sub> icebox as soon as they reached the laboratory. Unfiltered washings with 150 to 500 units of penicillin added were inoculated. Various combinations of amniotic and allantoic passages in eggs with or without mouse passages were used. In some instances evidence suggesting the possible presence of influenza virus was obtained in the course of some of the early passages in the embryonated eggs by the hemagglutination of the embryonic erythrocytes, but subsequent passages failed to establish the presence of a virus in most of them. Successful isolations of influenza viruses from this season's cases had been obtained only from the three cases mentioned at the time the present studies were being carried out. The three strains were readily identified serologically as type B influenza and were used in the present studies for comparison with the Lee strain.

*Cases Studied.*—Included in the serologic study are eleven families and two isolated cases, a total of thirty-nine persons. In each family there was at least one with typical symptoms of clinical influenza of moderate severity and from one to four others, either patients or healthy family contacts, from whom sera for serologic studies were obtained at the same time. The two isolated cases included a pupil and a cafeteria worker at the Junior High School. The influenza antibody titers in each of these individuals are shown in Table I, together with the date of onset and a rough estimate of severity in the cases of clinical influenza. Each titer represents the average for all the tests done with the virus and serum concerned. Patients in whom the symptoms were essentially those of a mild common cold with little or no fever and with coryza as the only significant feature are listed under Severity as *C*; those with low grade fever and diarrhea as the outstanding manifestation are designated as *D* in the same column in Table I.

*Serologic Results in Typical Cases of Influenza.*—

*Influenza A Antibodies:* There were nineteen characteristic cases of clinical influenza, and the symptoms in each instance began between Dec. 9 and 15. There were three such cases in each of two families, two in each of three families, and six that were the only typical cases among the persons studied in their families; there was one isolated case of clinical influenza with no family contacts included in the study. In none of these nineteen cases was there any significant rise in titer of antibodies against the PRS strain of influenza A by either the agglutinin inhibition or complement fixation test.

The level of the antibody titers obtained with the PRS strain by the tests employed are worth noting. A titer of 32 or higher was obtained in only one of the sera (the convalescent serum of Patient 7) by the complement fixation test, whereas titers ranging from 64 to 384 were obtained by the agglutinin inhibition test in both of the sera from six individuals and in the acute serum of a seventh person. The low titers were not the result of poor sensitivity of the

TABLE I. RESULTS OF SEROLOGIC TESTS

PATIENT	FAMILY	SEX	AGE	CLINICAL INFLUENZA		DATE OF ONSET	SEVERITY	DATE OF SERUM	AVERAGE TITER OF INFLUENZA ANTIBODIES									
									PFS		LEE		WVO		MF		MB	
									AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION
1	F	M	15	Dec. 9	++	Dec. 14	++	Dec. 14	80	16	51	48	32	19	24	28	24	32
2		F	13	Dec. 30	-	Dec. 30	-	Dec. 30	72	12	597	363	384	216	256	320	384	448
3		F	49	--	-	Dec. 30	-	Dec. 14	48	3	14	6	16	10	12	10	32	6
4		M	57	--	-	Dec. 30	-	Dec. 14	24	3	24	15	16	28	16	28	64	24
5		M	75	--	-	Dec. 14	-	Dec. 14	32	3	13	9	16	14	8	14	4	10
6	Ba	F	12	Dec. 13	+++	Dec. 13	+++	Dec. 13	12	10	8	15	4	14	16	14	4	12
7		F	10	Dec. 9	++	Dec. 15	++	Dec. 15	6	2	6	9	4	20	3	14	4	20
8		F	9	--	-	Dec. 15	-	Dec. 15	28	3	9	12	4	16	8	16	2	14
9		F	42	Dec. 8	C*	Dec. 15	C*	Dec. 15	28	2	6	4	2	28	8	28	3	5
10	D	M	14	Dec. 10	+++	Dec. 13	+++	Dec. 13	8	2	6	7	2	5	3	3	3	3
11		F	40	--	-	Dec. 29	-	Dec. 29	24	9	20	14	8	12	24	16	24	16
12		M	43	Dec. 5	D†	Dec. 29	D†	Dec. 29	16	12	544	512	256	192	256	448	192	512
13		F	65	Dec. 15	+++	Dec. 30	+++	Dec. 30	32	16	28	16	4	14	6	16	16	10
14	Mc	M	13	Dec. 7	+++	Dec. 14	+++	Dec. 14	24	3	192	224	128	20	8	20	64	24
15		M	14	Dec. 12	++	Dec. 30	++	Dec. 30	48	4	80	52	64	64	32	96	192	192
16		M	14	Dec. 13	+++	Dec. 14	+++	Dec. 14	48	4	10	2	4	72	32	80	128	36
17		F	44	--	-	Dec. 30	-	Dec. 30	8	2	320	256	128	112	48	80		
18	Mo	F	17	Dec. 10	+++	Dec. 13	+++	Dec. 13	48	11	6	44	4	32	16	40	4	48

to	M	H	Dec. 26	D	Dec. 16	16	12	7	13	4	10	8	8	4	20
20	F	40	--	-	Dec. 16	16	16	8	17	4	12	12	12	4	24
21	H	14	Dec. 11	+++	Dec. 30	16	10	12	32	2	20	3	3	4	24
22	F	38	Nov. 1	C	Dec. 29	16	8	3	6	16	12	10	18	2	7
23	M	40	--	-	Dec. 14	32	12	16	7	6	7	6	14	2	5
24	M	10	Dec. 12	++	Dec. 30	12	2	10	11	12	12	8	12	4	20
25	F	14	Dec. 13	++	Dec. 16	128	2	48	2	4	2	16	7	24	3
26	M	50	Dec. 10	+	Dec. 30	128	10	32	6	16	7	16	7	48	20
27	F	11	Dec. 15	+++	Dec. 15	64	8	3	12	5	10	2	14	24	12
28	F	40	Dec. 10	C	Dec. 15	72	7	6	16	2	16	2	20	8	20
29	M	41	Dec. 12	D	Dec. 30	8	2	3	16	2	16	2	10	3	24
30	M	15	Dec. 8	+++	Dec. 13	32	16	6	256	2	2	2	2	8	8
31	F	41	--	-	Dec. 16	8	5	5	4	320	256	128	224	256	512
32	M	47	Dec. 6	D	Dec. 20	4	3	14	4	2	5	4	4	4	3
33	N	13	Dec. 11	+++	Dec. 16	20	30	7	21	2	960	2	2	32	80
34	P	36	--	-	Dec. 16	4	14	4	512	512	4	2	5	6	20
35	M	37	--	-	Dec. 29	8	10	5	7	2	4	2	6	6	5
36	H	11	Dec. 10	+++	Dec. 15	48	4	16	15	16	24	16	3	8	3
37	P	46	Dec. 11	++	Dec. 29	48	4	10	160	128	256	128	256	96	20
38	P	46	Dec. 13	C	Dec. 14	12	2	10	6	2	7	6	8	8	14
39	T	13	Dec. 10	++	Dec. 13	24	14	6	256	48	128	32	5	6	2
					Dec. 30	24	10	04	4	2	20	2	10	6	3

\*C, Common cold.

†D, Diarrhea and fever.

‡Ac, Anticomplementary.

strain, since high titers and sharp rises in titers to high levels were obtained with the same antigen by both tests in unvaccinated individuals and in several patients with influenza A which occurred late in January, 1946.<sup>5</sup>

*Influenza B Antibodies:* Significant rises in titer of influenza B antibodies were demonstrated with two or more of the four strains used in fourteen of these cases and the rises were quite marked in most instances. Two additional cases showed significantly high titers but no rises were demonstrated. In Patient 14 the initial blood was obtained on the seventh day after the onset of symptoms, and while no significant rise in titer of antibodies for any of the type B strains was demonstrated, the titers of these antibodies initially were comparable with or higher than the titers obtained with the convalescent phase sera in several other cases in which a fourfold or greater rise had been demonstrated. This suggests the likelihood that a maximum titer already had been reached in that case at the time when the initial blood was drawn. Twin siblings of this case each showed a marked rise in influenza B antibodies but the onset of their symptoms occurred five and six days later, respectively, and their initial titers were much lower. In Patient 13 only a single sample of blood was obtained fourteen days after the onset of symptoms and in that serum high titers of antibodies against all of the influenza B strains were obtained by both tests. These titers were comparable with the convalescent titers in cases exhibiting a marked rise, indicating that in this case too the high titers were probably the result of recent infection with influenza B.

*Negative Serologic Responses:* The three remaining typical cases of clinical influenza (Patients 24 to 26) showed no significant titers or rises in titers of influenza B antibodies. The patients were all members of Family W and were the only persons studied in that family. The initial sera in these cases were obtained between the third and sixth days after the onset of clinical influenza. The illness in these patients occurred during the peak of the outbreak and was considered to be of average severity in two and mild in the third. The antibody titers obtained with the Lee strain and with two or all three of the recently isolated strains of influenza B were low in almost every instance. The initial titers of antibodies for PR8 were moderately high in the agglutinin inhibition tests in two of these cases but the complement-fixing antibodies were of low titer for this strain in both instances. Both tests gave low titers with the PR8 strain in the third case.

*Strain Differences:* A few details concerning the influenza B antibodies in the typical cases of clinical influenza are worth noting. In every case in which a rise in titer of antibodies was demonstrated with the Lee strain, comparable rises in titer of antibodies also were demonstrated with the recently isolated strains. There were three cases, however, in which tests with the Lee strain failed to bring out a rise in titer of influenza B antibodies by either agglutinin inhibition or complement fixation or by both tests when such a rise was demonstrated with the recently isolated strains. The original titers of influenza B antibodies in these cases were all low. The sera in Patient 18 showed no rise in titer of agglutinin inhibiting antibodies for the Lee virus when tested on

three separate occasions; however, a significant rise in titer was demonstrated by the complement fixation tests simultaneously with the same virus, and dilutions of serum made up at the same time. Tests done with the WC and MF strains also showed two- to fourfold rises in titer in this case, but the results with the epidemic strain MB were similar to those obtained with the Lee strain. The sera in Patients 21 and 27 showed no rise in titer of antibodies for the Lee strain by either the agglutinin inhibition or complement fixation tests done on four separate occasions, but they showed appreciable rises by both tests done with the WC and MF strains. In neither of these two was any significant rise elicited with the MB strain either. There were no gross discrepancies between the results obtained in the same sera with the WC and MF strains by either agglutination inhibition and complement fixation tests, and the differences that were noted were quantitative and were usually only slight. The findings in these three cases suggest that the WC and MF strains are more closely related to each other than to the Lee and MB strains and also that the MB strain is more nearly like the Lee strain than are the other two recently isolated strains. Such a deduction, however, requires substantiation from more detailed antigenic studies before it is acceptable.

*Cases Resembling Common Cold.*—The four patients in whom fever was absent and the symptoms were considered to be those of the common cold are of interest. Three of them (Patients 9, 22, and 28) were mothers of families in which at least one child had clinical influenza associated with a significant rise in titer of antibodies for the type B viruses. The fourth (Patient 38) was an adult cafeteria worker at the Junior High School. The onset of symptoms in Patient 22 occurred early in November, 1945, but in the others the illness began at about the same time as in those patients with the typical course of clinical influenza. The sera of each were tested several times with the PR8 and Lee viruses and at least once with each of the three recently isolated strains. None of these four cases showed significantly elevated titers or rises in titers of antibodies for any of the viruses used. The initial titers, in fact, were similar to those obtained with the same viruses in the acute phase sera of cases of clinical influenza showing typical rises with the strains of influenza B.

*Patients With Intestinal Manifestations.*—The four patients in whom the symptoms were predominantly gastrointestinal are also of interest because of the possible relation of influenza virus infection to these symptoms. Each of these patients was the father of a family in which there was at least one child with clinical influenza in whom a rise in titer of antibodies for at least two of the strains of type B influenza virus was demonstrated. In none of these four was a significant rise in titer of antibodies demonstrated for any of the viruses used. The data on these four patients, however, are not so clear as in the group with the symptoms of colds. In one of them, Patient 29, there was only a single specimen of serum obtained eighteen days after the onset of symptoms and all of the titers were low. In each of two others, Patients 12 and 32, the initial blood was obtained ten days after the onset of fever and diarrhea. In the latter two the titers of antibodies for some of the type B

strains, particularly the titers obtained by the complement fixation test, were higher than in most of the acute phase sera of patients in whom a significant rise was later demonstrated. Finally, in the fourth, Patient 19, the symptoms began ten days after the initial specimen was obtained and only four days before the second sample of blood was drawn.

*Household Contacts.*—The results of the tests on the sera of the healthy household contacts of those patients in whom a rise in titer of influenza B antibodies was demonstrated are also of interest since little information is available concerning the influenza antibody response under just these conditions. There were twelve such household contacts in whom pairs of blood samples were obtained at the same time, as in the patients with clinical influenza. Four of the twelve were in Family F and two in Family N, and there was one in each of six other families. Unfortunately only two of these contacts were children. The rest were adults; almost all of them were parents of the patients, presumably being in fairly close contact with them. That was particularly true of mothers who in each instance nursed their children through the illness at home.

Half of these healthy contacts showed no rise in titer of antibodies against any of the four type B strains by either of the tests used. There were two individuals, Patients 2 and 5, both in Family F, in whose sera a fourfold rise in titer was elicited against the epidemic strain MB by the complement fixation test. In both of them the agglutinin inhibition test showed a twofold rise with the same virus, and similar slight rises which are not ordinarily considered to be significant also were noted in the complement fixation tests with the remaining type B strains. Four other contacts (Patients 3, 8, 11 and 17) each showed a twofold rise in titer of antibodies against one or more of the type B strains by either or both tests. There were only two instances (Patients 17 and 34) in which a twofold rise in titer was elicited with the PR8 strain by one or the other of the tests. Except for the elevated titers of agglutinin inhibition with PR8 mentioned earlier, the titers in the initial samples in all of these healthy contacts were essentially similar to those observed in the acute phase sera of the patients with clinical influenza in whom significant rises in influenza B antibodies were elicited.

*Comparison of Agglutinin Inhibition and Complement Fixation Tests.*—Except as already noted, the levels of the antibody titers obtained in any single specimen of serum by inhibition of hemagglutination and by complement fixation of the same virus usually corresponded fairly closely considering the limitations of the methods. The greatest and most frequent discrepancies were observed in the titers obtained with the PR8 strain: Several sera yielded high titers by inhibition of agglutination with this strain, but significantly high titers with the complement fixation tests were not observed. Except as noted earlier, the two tests also gave quite comparable titers in the same sera with each of the four strains of influenza B virus. In a few sera slightly higher titers with these viruses were obtained by complement fixation than by the inhibition of hemagglutination.

*Tests With the BON Strain of Influenza B.*—After all of these studies were completed, Dr. John F. Enders suggested that serologic tests with the BON strain also might be of interest. This strain was isolated by chick-embryo inoculation in Australia in 1943 by Beveridge and associates,<sup>6</sup> who showed it to be serologically related to, but readily distinguishable from, the Lee strain of influenza B. The results of the tests done on fifteen pairs of sera that were still available are shown in Table II.

TABLE II. RESULTS OF SEROLOGIC TESTS WITH STRAIN BON

PATIENT	ANTIBODY TITERS			
	AGGLUTININ INHIBITION		COMPLEMENT FIXATION	
	FIRST SPECIMEN	SECOND SPECIMEN	FIRST SPECIMEN	SECOND SPECIMEN
1	48	512	192Ac*	448
2	12	16	12	24Ac
4	8	6	16	10
7	8	160	12	576
8	6	8	5	6
9	6	6	10	5
10	12	512	10	768
11	16	16	32	20
12	16	12	40Ac	20
20	24	16	20	20
21	3	16	2	24
22	16	12	6	5
25	32	32	12	12
26	8	8	10	20
37	8	48	20	96

\*Ac, Anticomplementary in lower dilutions.

Of particular interest is the demonstration of definite rises in antibody titers against the BON strain in Patient 21. In this respect the BON strain was similar to the WC and MF strains and unlike the Lee and MB strains. Tests were also done on the sera of six other patients with influenza including those from four (Patients 1, 7, 10, and 37) in whom definite rises in antibody titers had been elicited with the other type B strains and those from two others (Patients 25 and 26) in whom rises in antibody titers were not demonstrated. Similar results were obtained with the BON strain in each instance. The remaining eight persons whose sera were tested included two who were considered to have "colds" (Patients 9 and 22), one who had diarrhea (Patient 12), and five healthy contacts. The results obtained with the BON strain in the sera of these individuals also were essentially the same as those obtained with the other type B strains.

#### DISCUSSION

Among the present cases the serologic evidence clearly indicates that influenza B was the predominant if not the only virus implicated in the influenza outbreak at Needham. Evidence suggesting that probably only type B influenza occurred during the same period in Boston has also been obtained serologically and from the only successful isolation of virus from patients with acute influenza. Similar experiences were also reported from other localities at the same time.<sup>7, 8</sup> In a boys' school located several miles north of Boston, however,



an outbreak occurred during January, 1946, which was proved serologically to be due to influenza A, and no cases of influenza B infection were identified among students at that school.<sup>9</sup> Furthermore, among sporadic cases of clinical influenza which occurred in Boston a few weeks after the peak of epidemic prevalence of influenza B, several were identified serologically as influenza A. Included among the latter were persons who had previously suffered an illness, which was proved serologically to be influenza B during the epidemic, and were thus instances of consecutive infections with two distinct viruses.<sup>2</sup> Isolation of influenza A virus during December, 1945, was reported from Illinois,<sup>10</sup> and both influenza A and B infections were demonstrated in Australia earlier that year.<sup>11</sup>

The findings in Family W in which three seemingly typical cases of clinical influenza yielded negative serologic results have not been explained. These cases occurred during the height of the epidemic and one of them was in a pupil at the Junior High School. The cases may have been somewhat less severe than most of the others that were studied. Since a virus was not isolated from any of these cases, the possibility cannot be excluded that they had responded in a highly specific manner to their own strain but not to the related strains of influenza B virus used in this study. On the other hand, they may represent infections with a totally different virus or, what seems more likely, failures to respond with a rise in antibodies to typical strains, as Magill and Sugg<sup>12</sup> have shown in some cases of influenza A infection.

During the present outbreak in Needham, Mass., as well as in Boston and elsewhere, there were many patients with predominantly gastrointestinal symptoms interspersed among those with epidemic influenza. Some of these patients with so-called "intestinal flu" also had fever and other systemic symptoms such as malaise, headache, and prostration, which made it difficult to exclude the diagnosis of clinical influenza. The four cases included in this study and several others which were studied in Boston during the same time all failed to yield any definite serologic evidence of influenza by the development of antibodies to any of the viruses used in this study, in spite of close association of most of them with proved cases of influenza B.

Cases of common cold and other undifferentiated respiratory infections are always prevalent during the season when influenza outbreaks occur, and it is not always possible to distinguish isolated cases from those of influenza on purely clinical grounds. In the present study the four cases which clinically were considered to be common colds all failed to respond to their infection with a rise in antibodies to any of the viruses used, and the same was true of several additional cases studied in this laboratory during the same period. There were other cases, however, in which clinical differentiation was difficult and some of them proved serologically to be influenza B. The number of individuals of this type and of those with intestinal manifestations was too small to warrant any final deductions as to their relation to the prevalent influenza virus infections.

The same also may be said of the observations made on influenza antibodies in the healthy family contacts of patients with proved influenza B. The

present studies reveal no clear evidence of inapparent infection in these individuals, unless the slight rises in antibody titers can be interpreted in that way. Nor was any evidence found to indicate that the healthy contacts were protected by virtue of an adequate antibody content in their blood. These negative results may be a reflection of a type of resistance to infection among these contacts which could not be measured serologically with the available viruses or they may indicate an inapparent infection with an inadequate antibody response or with a strain of virus having low infectivity or low antigenicity in these individuals. Inapparent infections with influenza A as manifested by the development of antibodies in the absence of symptoms have been reported,<sup>13-16</sup> and influenza A virus has been isolated from apparently normal human contacts during an epidemic of influenza.<sup>16</sup> Serologic tests on contacts of patients with influenza B infections in an institutional outbreak in 1939 indicated an incidence of subclinical infection equal to that of clinical infections.<sup>17</sup>

Evidence was obtained which suggested antigenic differences between the recently isolated strains and the Lee strain of influenza B. Some cases which failed to show a rise to the latter reacted with a characteristic antibody response to some of the former. On the basis of the very meager evidence on Patient 21, together with the results on Patients 18 and 27, the strain MB from the Needham case appears to be antigenically similar to the Lee virus, whereas the strains WC and MF are more like the BON strain. There also was suggestive evidence of discrepancies between the antibody titers obtained by inhibition of hemagglutination and by complement fixation with the same viruses. The discrepancies were more frequent and more consistent (that is, the inhibition titers were always much higher) with the PR8 strain than with the B strains. With the latter viruses some titers were higher with one test and some with the other. These may be related in some way to the differences in antigenic components of the viruses as described by Wiener and associates.<sup>18</sup>

#### SUMMARY AND CONCLUSIONS

1. Serologic studies were carried out in cases and in family contacts during an outbreak of influenza which occurred in Needham, Mass., during mid-December, 1945.
2. The typical cases of influenza, with three exceptions, yielded evidence of infection with influenza B by a rise in titer of antibodies. The three exceptions were all individuals in the same family.
3. Some of the cases showed a rise in antibodies when tested with two strains of influenza B recently isolated from cases in Boston but failed to show such a rise in the same sera when tested with the Lee strain of influenza B and with a strain isolated from one of the cases in Needham.
4. Among the family contacts of the serologically proved cases of influenza B there were four who had symptoms which were more like those of the common cold (mostly coryza without fever) and four others who had low grade fever and predominantly gastrointestinal symptoms. No serologic evidence of influenza virus infection was obtained in any of these individuals.

5. Healthy family contacts of the proved cases of influenza B also failed to show significant rises in antibody titers in their sera for any of the strains of influenza B virus used or for the PR8 strain of influenza A, but slight rises were elicited in some of them.

6. There were some discrepancies between the titers obtained in the same sera by inhibition of hemagglutination and by complement fixation test with the same virus. These discrepancies were more frequent with the PR8 strain, and when they occurred, the agglutinin inhibition test with this virus gave the higher titers. Some sera gave higher titers in this test with the B strains, but more gave higher titers with the complement fixation test.

#### REFERENCES

1. Rubenstein, A. D., Ory, E. M., Meads, M., and Finland, M.: Influenza B in Needham, Massachusetts, December, 1945; *Epidemiological Studies in Cases and in Family Contacts*. To be published.
2. Francis, T., Jr., and Salk, J. E.: A Simplified Procedure for the Concentration and Purification of Influenza Virus, *Science* 96: 499-500, 1942.
3. Hirst, G. K.: Adsorption of Influenza Hemagglutinins and Virus by Red Blood Cells, *J. Exper. Med.* 76: 195-209, 1942.
4. Enders, J. F., Kane, L. W., Cohen, S., and Levens, J. H.: Immunity in Mumps. I. Experiments With Monkeys (*Macacus mulatta*). The Development of Complement-Fixing Antibody Following Infection and Experiments on Immunization by Means of Inactivated Virus and Convalescent Human Serum, *J. Exper. Med.* 81: 93-117, 1945.
5. Finland, M.: Unpublished data.
6. Beveridge, W. I. B., Burnet, F. M., and Williams, S. E.: The Isolation of Influenza A and B Viruses by Chick-Embryo Inoculation, *Australian J. Exper. Biol. & M. Sc.* 22: 1-7, 1944.
7. Francis, T., Jr., Salk, J. E., and Brace, W. M.: The Protective Effect of Vaccination Against Epidemic Influenza B, *J. A. M. A.* 131: 275-278, 1946.
8. Anonymous: Prevalence of Communicable Diseases in the United States, Dec. 2 to 29, 1945, *Pub. Health Rep.* 61: 109-114, 1946.
9. Ingalls, T. H., Gallagher, J. R., and Enders, J. F.: Outbreak of Influenza A in a Boys' School During January, 1946, *New England J. Med.* 235: 786-788, 1946.
10. Foster, G. F., and Shaughnessy, H. J.: Isolation of Type A Influenza Virus in Recent Epidemic in Chicago Area, *Science* 104: 208-209, 1946.
11. Burnet, F. M., Stone, J. D., and Anderson, S. G.: An Epidemic of Influenza B in Australia, *Lancet* 1: 807-811, 1946.
12. Magill, T. P., and Sugg, J. Y.: The Significance of Antigenic Differences Among Strains of the 'A Group' of Influenza Virus, *J. Exper. Med.* 80: 1-7, 1944.
13. Francis, T., Jr., Magill, T. P., Rickard, E. R., and Beck, D. M.: Etiological and Serological Studies in Epidemic Influenza, *Am. J. Pub. Health* 27: 1141-1160, 1937.
14. Stuart-Harris, C. H., Andrewes, C. H., Smith, W., Chalmers, D. K. M., Cowen, E. G. H., and Hughes, D. L.: A Study of Epidemic Influenza: With Special Reference to the 1936-7 Epidemic. Medical Research Council Special Report Series, No. 228, London, 1938, His Majesty's Stationery Office.
15. Rickard, E. R., Lennette, E. H., and Horsfall, F. L., Jr.: Comprehensive Study of Influenza in Rural Community, *Pub. Health Rep.* 55: 2146-2167, 1940.
16. Crowley, J. H., Thigpen, M. P., and Rickard, E. R.: Isolation of Influenza A Virus From Normal Human Contacts During an Epidemic of Influenza A, *Proc. Soc. Exper. Biol. & Med.* 57: 354-356, 1944.
17. Nigg, C., Eklund, C. M., Wilson, D. E., and Crowley, J. H.: Study of an Epidemic of Influenza B, *Am. J. Hyg.* 35: 265-284, 1942.
18. Wiener, M., Henle, W., and Henle, G.: Studies on the Complement Fixation Antigens of Influenza Viruses Types A and B, *J. Exper. Med.* 83: 259-279, 1946.

## THE EFFECT OF ANTIHISTAMINE DRUGS UPON SERUM-INDUCED MYOCARDITIS IN RABBITS

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THE recent work of Rich and Gregory<sup>7</sup> upon the role of hypersensitivity in rheumatic fever once again has focused attention upon a problem that has perplexed investigators for many years. Before discussing the work to be presented in this paper, it would seem advisable to review briefly some of the pertinent contributions on this subject.

Swift<sup>10</sup> pointed out in a classic paper in which a complete review of the literature is given that the histologic picture of rheumatic fever may vary in different individuals, just as does the clinical picture, and that a patient who has had a rheumatic infection reacts more profoundly to the etiologic agent than does the individual who never has had rheumatic fever. He believes that this reaction is the result of a state of hypersensitivity. In a later communication Swift and co-workers<sup>11</sup> describe attempts to diminish the state of hypersensitiveness in rheumatic fever by giving repeated injections of a specific streptococcal vaccine. These workers believe that "if by this means of immunization the natural resistance of the tissues can be so enhanced that subsequent infection is met and tolerated with only a minimum of injury, the method will have been justified."

Gross and associates<sup>4</sup> attempted to produce rheumatic-like lesions by injecting various strains of streptococci isolated from patients with rheumatic fever into experimental animals. Their criteria for a diagnosis of experimental rheumatic fever were: (1) the Aschoff body, (2) nonbacterial pericarditis, and (3) nonbacterial verrucous endocarditis. These authors were unable to meet the criteria in their experimental lesions, and they stated that other workers had been too lax in their interpretation of results. The present paper also points out that perivascular accumulations occur in the normal rabbit heart.

Clark and Kaplan<sup>2</sup> presented a summary of the evidence that anaphylactic vascular lesions induced in animals are very similar to the histologic changes occurring spontaneously in rheumatic fever, periarteritis nodosa, and scarlet fever. The authors present the pathologic findings in two cases of serum sickness and suggest that the lesions are the result of a hypersensitive state. Rich<sup>6</sup> in a later paper again emphasized the role of anaphylactic hypersensitivity in patients who developed periarteritis nodosa following serum sickness.

In 1943 Rich and Gregory<sup>5</sup> published evidence that lesions resembling periarteritis nodosa can result from injections of sterile unpreserved horse serum into rabbits. In the same year work was presented in which lesions similar to rheumatic carditis were produced in rabbits by a similar method.

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Rich and Gregory<sup>7</sup> state that "the valvular and myocardial lesions which in their situation and basic characteristics conform so closely with those of rheumatic carditis that we believe they may provide a fair measure of support for the view that the specific rheumatic lesions may themselves be manifestations of an anaphylactic hypersensitive reaction." In this study eleven out of thirty-six rabbits developed rheumatic-like changes in the hearts. Rich and Gregory<sup>7</sup> describe in detail the characteristic cardiac lesions of rheumatic carditis and discuss ten major points as evidence that rheumatic lesions are hypersensitive in character. The histologic changes which were produced by Rich and Gregory were: (1) lesions similar to Aschoff bodies with the presence of Anitschkow myocytes or cells similar to them, (2) focal accumulations of mononuclear cells and large basophilic cells similar to those that characterize the Aschoff body, and (3) nonspecific lesions consisting of edema, round cells, and polymorphonuclear leucocytes.

Fox and Jones<sup>8</sup> repeated the work of Rich and Gregory and produced lesions similar to those of rheumatic carditis in twenty out of thirty rabbits. Robinson<sup>9</sup> was unable to produce cardiac lesions in ten rabbits injected with sterile horse serum; however, it must be noted that the animals in this work were killed in seven to twenty days, which probably accounts for the result obtained.

In 1945 Aikawa<sup>1</sup> summarized very completely the literature on this entire subject and discussed many papers which have not been mentioned in this presentation.

#### PRESENT STUDY

In the work herein reported the primary consideration has been to produce lesions in the rabbit heart according to the method of Rich and Gregory<sup>8</sup> and to note the effect of certain drugs upon the development of such lesions. No claim is made that the lesions are characteristic of rheumatic fever, although in many instances the changes produced bear a striking resemblance to the histologic picture of acute rheumatic carditis.

Thirty-nine young adult rabbits were used in our study. Of this group, nine animals died before the experiment was completed and the hearts of three of these nine were examined. However, our results are compiled only on those animals that lived throughout the entire study.

Sterile unpreserved horse serum was injected intravenously into rabbits according to the method of Rich and Gregory.<sup>8</sup> In one group of animals serum with preservative was used, and this fact will be emphasized in the results presented herein.

The animals were divided into the following groups:

Group I. Twelve rabbits received serum alone.

Group II. Nine rabbits received serum plus 2 mg. of beta dimethylaminoethyl benzhydryl ether hydrochloride (benadryl) two times daily subcutaneously throughout the duration of the experiment.

Group III. Six rabbits received serum plus 10 mg. of benadryl per kilogram of body weight two times daily subcutaneously throughout the duration of the experiment.

Group IV. Nine animals received serum plus an experimental drug\* which has been shown to have antihistamine qualities, as measured by the Loew histamine spray test and by giving protection against anaphylaxis in guinea pigs. Throughout the remainder of this paper the drug will be named No. 1627. The dosage was 3 mg. per kilogram two times daily subcutaneously.

Group V. Three rabbits received serum alone and were used as controls for Group IV. In Group IV and V the third, or shocking, dose of serum contained a small amount of preservative, the significance of which is not known. For this reason the series of controls is kept in a separate group.

### RESULTS

The results are summarized in Tables I to V.

Group I. Serum alone. Ten rabbits developed marked cardiac lesions. One rabbit developed moderate lesions. These consisted of the following types of changes:

1. Pericapillary and periarteriolar collections of lymphocytes and monocytes with occasional polymorphonuclear leucocytes. In many sections small areas of necrosis were found adjacent to blood vessels, surrounded with mononuclear cells, which resembled the Aschoff body. In many vessels the entire wall of the vessel was infiltrated with cells. Endothelial proliferation was marked, and in some sections hyalinization of the vessel wall was evident. Periarteriolar proliferations of fibroblasts frequently were present.

2. Scattered areas of hyalinization of muscle fibers.

3. Loose infiltrations with small round cells scattered between muscle fibers.

4. Occasional areas of vacuolar degeneration of fibers.

These changes are demonstrated in Figs. 1 to 6.

Group II. Serum plus benadryl. Four rabbits revealed no lesions or minimal changes. Two rabbits developed moderate cardiac lesions. In this

TABLE I. GROUP I SERUM ALONE

RABBIT	WEIGHT IN GRAMS	DURATION OF EXPERIMENT (DAYS)	RHEUMATOID LESIONS
1	1,939	29	Marked
2	1,591	29	Marked
3	1,932	29	Marked
4	2,433	29	Marked
5	2,251	28	Marked
6	3,400	28	Animal died; not examined
7	3,747	27	Marked
642	2,000	29	Marked
643	2,000	29	Marked
644	2,500	29	Marked
645	2,450	29	Marked
647	1,700	29	Moderate
			Marked

\*Developed by G. D. Searle & Company, Chicago, Ill.

group the absence of perivascular changes was striking. Only in an occasional section were lesions found adjacent to blood vessels. No lesions resembling Aschoff bodies were seen. There were present, scattered between muscle fibers, small infiltrations of mononuclear cells and lymphocytes. Occasional areas of vacuolar degeneration were seen. Throughout all sections the lesions were very much less marked than those of Group I.



Fig. 1.

Fig. 1.—Arteriole with vacuolization of endothelium, hyperplasia, and swelling of media and surrounding dense infiltration of lymphocytes and polymorphonuclear leucocytes ( $\times 540$ ).



Fig. 2.

Fig. 2.—Periarterial infiltration with lymphocytes and monocytes ( $\times 200$ ).

Group III. Serum plus 10 mg. of benadryl per kilogram twice daily. Three rabbits showed no cardiac changes. Two animals revealed the presence of only an occasional perivascular infiltration, and these lesions were classified as minimal. One rabbit did develop a moderate number of lesions but not nearly as marked as in the control group.

Group IV. Serum plus drug No. 1627. Eight rabbits developed no lesions or only very slight changes. One rabbit developed moderate changes. Only occasional perivascular lesions were present. Nothing resembling an Aschoff body was seen. It must be pointed out that in this group and in Group V the third dose of serum contained a small amount of preservative.

TABLE II. GROUP II SERUM PLUS BENADRYL (2 MG. TWICE DAILY)

RABBIT	WEIGHT IN GRAMS	DURATION OF EXPERIMENT (DAYS)	RHEUMATOID LESIONS
8	1,933	31	None
9	1,800	31	None
10	2,240	31	Moderate
11	1,962	4/28/46 to ?	Animal died; not examined
12	1,700	4/28/46 to ?	Animal died; not examined
13	2,005	4/28/46 to ?	Animal died; not examined
14	2,580	30	Very slight
15	2,430	30	Moderate
16	2,500	30	None

3

TABLE III. GROUP III SERUM PLUS BENADRYL (10 MG. PER KILOGRAM TWICE DAILY)

RABBIT	WEIGHT IN GRAMS	DURATION OF EXPERIMENT (DAYS)	RHEUMATOID LESIONS
636	1,600	29	No lesion
637	2,400	29	No lesion
638	1,850	29	Moderate
639	1,600	29	No lesion
640	1,700	29	Minimal
641	2,200	29	Minimal

TABLE IV. GROUP IV SERUM PLUS DRUG NO. 1627

RABBIT	DURATION OF EXPERIMENT (DAYS)	RHEUMATOID LESIONS
504	26	Very slight
505	26	Moderate
506	26	Very slight
507	26	Very slight
512	26	Very slight
513	26	None
515	9/ 3/46 to 9/20/46	Animal died; no lesions
576	9/ 3/46 to 9/20/46	Animal died; no lesions
577	26	Very slight

TABLE V. GROUP V SERUM ALONE

RABBIT	DURATION OF EXPERIMENT (DAYS)	RHEUMATOID LESIONS
578	26	Very slight
579	26	Marked
580	26	Marked



Group V. Serum alone to act as controls for Group IV. Two rabbits developed marked cardiac changes and one animal showed only slight changes.

#### DISCUSSION

From the literature reviewed and from the results herein reported, one is justified in stating that cardiac lesions may be produced in rabbits by the proper intravenous administration of horse serum. Gross and co-workers<sup>4</sup> state that perivascular infiltrations may occur in normal rabbits. In the present study

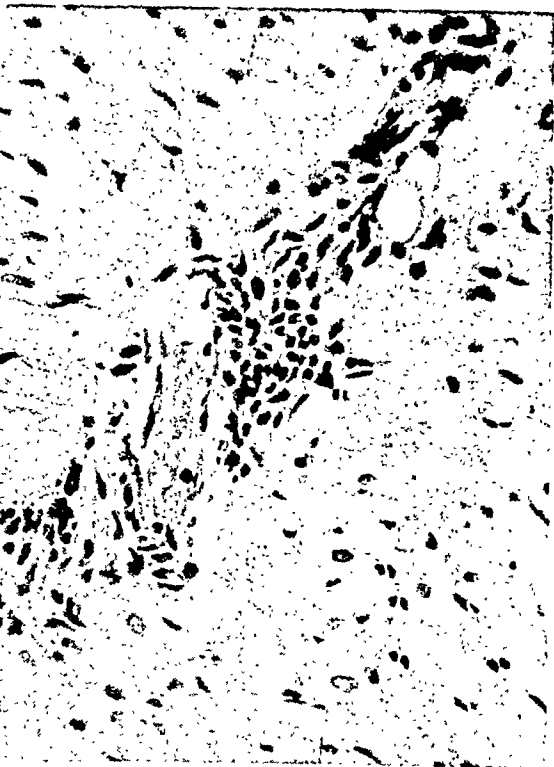


Fig. 3.

Fig. 3.—Lesion simulating an Aschoff body adjacent to arteriole ( $\times 450$ ).

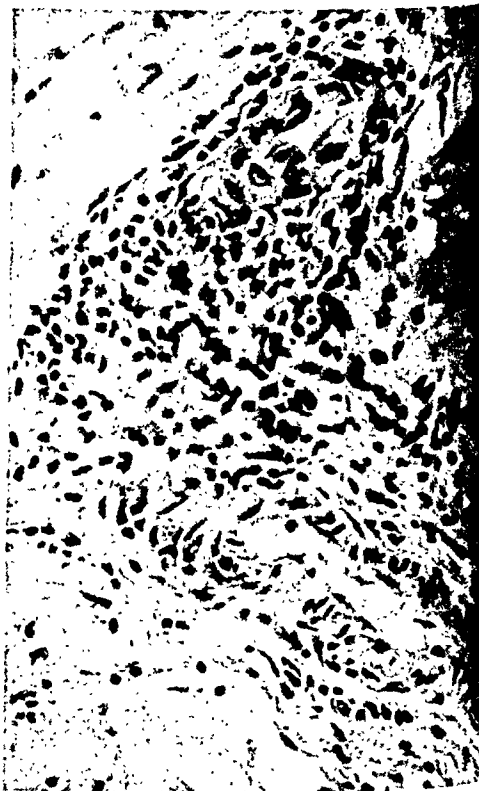


Fig. 4.

Fig. 4.—Lesion simulating an Aschoff body adjacent to arteriole ( $\times 540$ ).

only a very occasional perivascular lesion was found in the animals that received benadryl and drug No. 1627. The control animals showed rather extensive lesions. We therefore feel that even if perivascular lesions do occasionally occur in the normal rabbit heart this fact does not alter the results of this experiment. Miller<sup>5</sup> has stated that 60 per cent of normal rabbit hearts will show cellular infiltrations between the muscle fibers. This may be true and, if so, will in no way change the evaluation of the results of this work, since most of our emphasis has been placed on the presence of vascular and perivascular lesions.

The similarity of the changes found in our control groups to the cardiac lesions of acute rheumatic carditis is striking. Since two antihistamine drugs have been shown to impede the development of these cardiac lesions in rabbits, it would seem advisable for further experiments to be carried out in an effort to determine the relationship of rheumatic fever to a hypersensitive state.

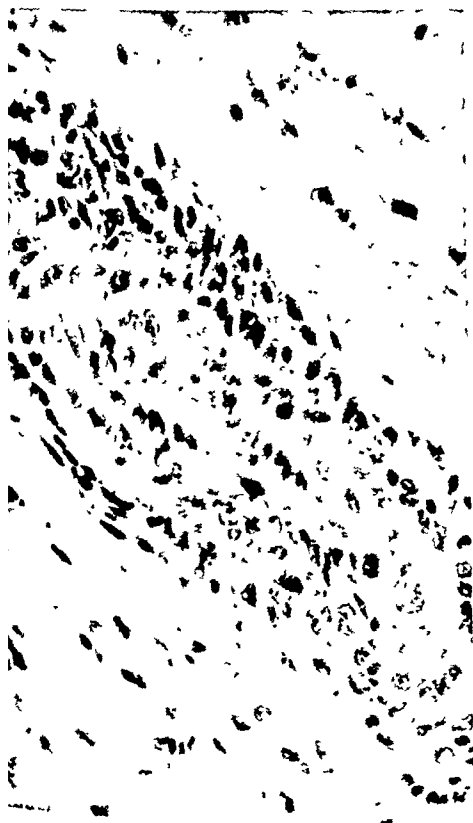


Fig. 5.



Fig. 6.

Fig. 5.—Infiltration around and into wall of artery by lymphocytes, monocytes, and eosinophiles; organizing thrombus is present in lumen of vessel ( $\times 540$ ).

Fig. 6.—Periaarteriolar infiltration ( $\times 500$ ).

#### SUMMARY

Evidence is presented that benadryl and drug No. 1627, two antihistamine drugs, will impede the development of serum-induced myocardial lesions in rabbits. A brief review of the pertinent literature is included.

#### CONCLUSIONS

1. Myocardial lesions may be produced in rabbits by the injection of sterile horse serum according to the method of Rich and Gregory.
2. Benadryl and drug No. 1627 are effective in impeding the development of these lesions.

We wish to acknowledge the cooperation and help of G. D. Searle & Company, and of Dr. Irwin C. Winters of that company, for supplying drugs and animals for part of this work, and also Parke, Davis & Company, Detroit, Mich., for supplying the benadryl used in this investigation.

## REFERENCES

1. Aikawa, J. K.: Hypersensitivity and Rheumatic Fever, *Ann. Int. Med.* 23: 969, 1945.
2. Clark, E., and Kaplan, B.: Endocardial, Arterial, and Other Mesenchymal Alterations Associated With Serum Disease in Man, *Arch. Path.* 24: 458, 1937.
3. Fox, R., and Jones, L.: Vascular Pathology in Rabbits Following Administration of Foreign Protein *Proc. Soc. Exper. Biol. & Med.* 55: 294, 1944.
4. Gross, L., Loewe, L., and Eliasoph, B.: Attempts to Reproduce Rheumatic Fever in Animals, *J. Exper. Med.* 50: 41, 1929.
5. Miller, C. P., Jr.: Spontaneous Interstitial Myocarditis in Rabbits, *J. Exper. Med.* 40: 543, 1924.
6. Rich, A. R.: The Role of Hypersensitivity in Periarthritis Nodosa, *Bull. Johns Hopkins Hosp.* 71: 123, 1942.
7. Rich, A. R., and Gregory, J. E.: Experimental Evidence That Lesions With the Basic Characteristics of Rheumatic Carditis Can Result From Anaphylactic Hypersensitivity, *Bull. Johns Hopkins Hosp.* 73: 239, 1943.
8. Rich, A. R., and Gregory, J. E.: The Experimental Demonstration That Periarthritis Nodosa Is a Manifestation of Hypersensitivity, *Bull. Johns Hopkins Hosp.* 72: 65, 1943.
9. Robinson, J. J.: Some Experimental Aspects of Streptococcus Infection and Rheumatic Fever, *Arch. Pediat.* 62: 387, 1945.
10. Swift, H. F.: Rheumatic Fever, *J. A. M. A.* 92: 2071, 1929.
11. Swift, H. F., Hitchcock, C. H., Derick, C. L., and McEwen, C.: Intravenous Vaccination With Streptococci in Rheumatic Fever, *Am. J. M. Sc.* 181: 1, 1931.

# EVIDENCE FOR THE ACTIVITY OF A SECOND MEMBER OF THE VITAMIN M GROUP (FERMENTATION FACTOR) IN SPRUE

## A CASE REPORT

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### INTRODUCTION

IN THE group of substances<sup>1</sup> loosely referred to as "folic acid" there are at least three related chemical compounds: (1) pteroylglutamic acid<sup>2</sup> (PGA), which has been synthesized and widely employed clinically and which is identical with the liver *Lactobacillus casei* factor<sup>3</sup>; (2) pteroyldiglutamylglutamic acid,<sup>2</sup> an *L. casei* factor which was isolated from "a fermentation residue," and, hence, was termed "fermentation factor,"<sup>4</sup> and which has not yet been available for widespread clinical testing; and (3) pteroylhexaglutamylglutamic acid<sup>5</sup> or vitamin B<sub>12</sub> conjugate, isolated from yeast<sup>6</sup> and tested clinically in a limited number of patients.<sup>7-9, 14</sup> Our initial hypothesis that vitamin M deficiency in the monkey is the experimental analogue of sprue in man<sup>10, 11</sup> was based in part on the demonstrated activity of pure fermentation factor or pteroyldiglutamylglutamic acid in the vitamin M-deficient monkey and the demonstration of the activity of the related simpler compound, pteroylglutamic acid, in patients with sprue. It has since become evident that this simpler substance (PGA) possesses activity in the monkey.<sup>12, 13</sup> Suárez and co-workers<sup>14</sup> have reported a patient with sprue who was successfully treated with 4.9 mg. of pteroyltriglutamic acid daily intramuscularly. Castle and associates<sup>15</sup> reported that this material was inactive in patients with pernicious anemia, while Goldsmith<sup>16</sup> has found that it possessed some activity in a patient with nutritional macrocytic anemia. In a preliminary note, Spies<sup>17</sup> stated that a patient with pernicious anemia had exhibited a slight reticulocyte response following the administration of fermentation factor. We have had a sufficient quantity of isolated crystalline fermentation factor\* to test its activity in one patient with sprue. Our experience with this case supplies additional evidence for the hemopoietic activity of the factor in this latter condition. Inasmuch as this isolated product is available in but limited quantities and our present supply is exhausted, it appears worth while to report this single case.

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## CASE REPORT

A 52-year-old white woman entered Vanderbilt University Hospital Dec. 19, 1946, with the complaint of recurrent diarrhea of seven months' duration. There had been eight to twelve bowel movements daily for periods of several days to several weeks followed by intervals of four or five days free of diarrhea. The diarrhea was associated with lower abdominal cramping. The stools were described as watery to mushy, of increased bulk, yellow in color, and very foul in odor. Tarry stools had not been noted and gross blood had not been seen. There had been no jaundice, nausea, vomiting, or food intolerance. Moderate anorexia was present. Over the seven-month period there had occurred a weight loss of 80 pounds.

During the four months prior to admission the patient had noted progressive weakness, pallor, exertional dyspnea, and periodic soreness of the tongue. The weakness and dyspnea ultimately confined her to bed for the month preceding admission. There had been swelling of the lower extremities and the eyelids for three months and increasing grayish pigmentation of the hands, arms, and lower legs. No history of excessive blood loss was obtained. The patient's diet had consisted of milk, corn bread, turnip greens, and a few other vegetables; it had included very little lean meat.

Physical examination revealed an extremely ill woman lying flat in bed; she had marked pallor and pronounced generalized wasting of the subcutaneous and muscular tissues. She weighed 120 pounds (54.4 kilograms). The skin was loose, dry, scaly, and of a grayish appearance about the face, neck, arms, hands, and the lower portion of the legs. A few scattered petechiae were present. The hair was dry and without luster. The retinas revealed several small hemorrhagic areas both old and recent. The mucous membranes were pale. The mouth was edentulous. The filiform papillae were atrophic along the margins of the tongue. No abnormality of the lungs was detected. The heart was diffusely enlarged and a loud, low-pitched, blowing systolic murmur was audible over the entire precordium, with the point of maximum intensity in the fourth left interspace. The blood pressure was 98/60 and the pulse rate was 98 beats per minute. There was moderate thickening of the peripheral vessels. The abdomen was protuberant and some tenderness was present in both lower quadrants. The liver and spleen were not palpable and no abdominal masses were detected. Several small hemorrhoids were noted. Moderate pitting edema was present over the sacrum and the upper and lower extremities below the elbows and knees, respectively. The tendon reflexes were hyperactive. The ability to accomplish fine movements of the fingers was impaired and vibratory sense was diminished at the wrists and over the lower extremities. The plantar responses were flexor.

The data for the erythrocyte and leucocyte counts, reticulocytes, hemoglobin, and packed cell volume are given in Fig. 1. A differential count of the white blood cells showed the following percentages: segmented neutrophils, 79; lymphocytes, 14; monocytes, 5; band forms, 1; and eosinophiles, 1. The platelets were scarce and the red blood cells showed marked anisocytosis and poikilocytosis. Aspirated sternal bone marrow revealed 6 per cent megaloblasts and 16 per cent erythroblasts, a picture compatible with a diagnosis of sprue or pernicious anemia. Gastric analysis revealed a histamine refractory achlorhydria. Other laboratory findings included: icteric index, 5; serum albumin, 2.58 Gm. per 100 c.c.; serum globulin, 2.16 Gm. per 100 c.c.; serum calcium, 7.9 mg. per 100 c.c.; and serum phosphorus, 2.4 mg. per 100 c.c. The stool fat content was 62 per cent of the dry weight. Repeated stool examinations revealed no occult blood, ova, or parasites, and no pathogenic organisms were found upon culture. The erythrocyte fragility did not differ from that of the control. The tourniquet test produced a few scattered petechiae. Prothrombin time was 41 per cent of normal. A cephalin flocculation test was negative after twenty-four hours and the thymol turbidity test showed 5 units. Serum levels of vitamins were as follows: vitamin C, 0.1 mg. per 100 c.c.; vitamin A, 39 I.U. per 100 c.c.; carotene, 9 to 16  $\mu$ g per 100 c.c.; and tocopherol, 0.45 mg. per 100 cubic centimeters. The vitamin A tolerance test following the oral administration of 200,000 I.U. of vitamin A was as follows: three hours, 43 I.U. per 100 c.c.; five hours, 51 I.U. per 100 c.c.; and nine hours, 46 I.U. per 100 cubic centimeters. A

tocopherol tolerance<sup>18</sup> performed simultaneously with the vitamin A tolerance yielded a flat "curve" with values as follows: fasting, 0.45 mg.; three hours, 0.43 mg.; five hours, 0.38 mg.; and nine hours, 0.45 milligram. A glucose tolerance test was invalidated because of the accidental simultaneous subcutaneous administration of 1.5 mg. of histamine.

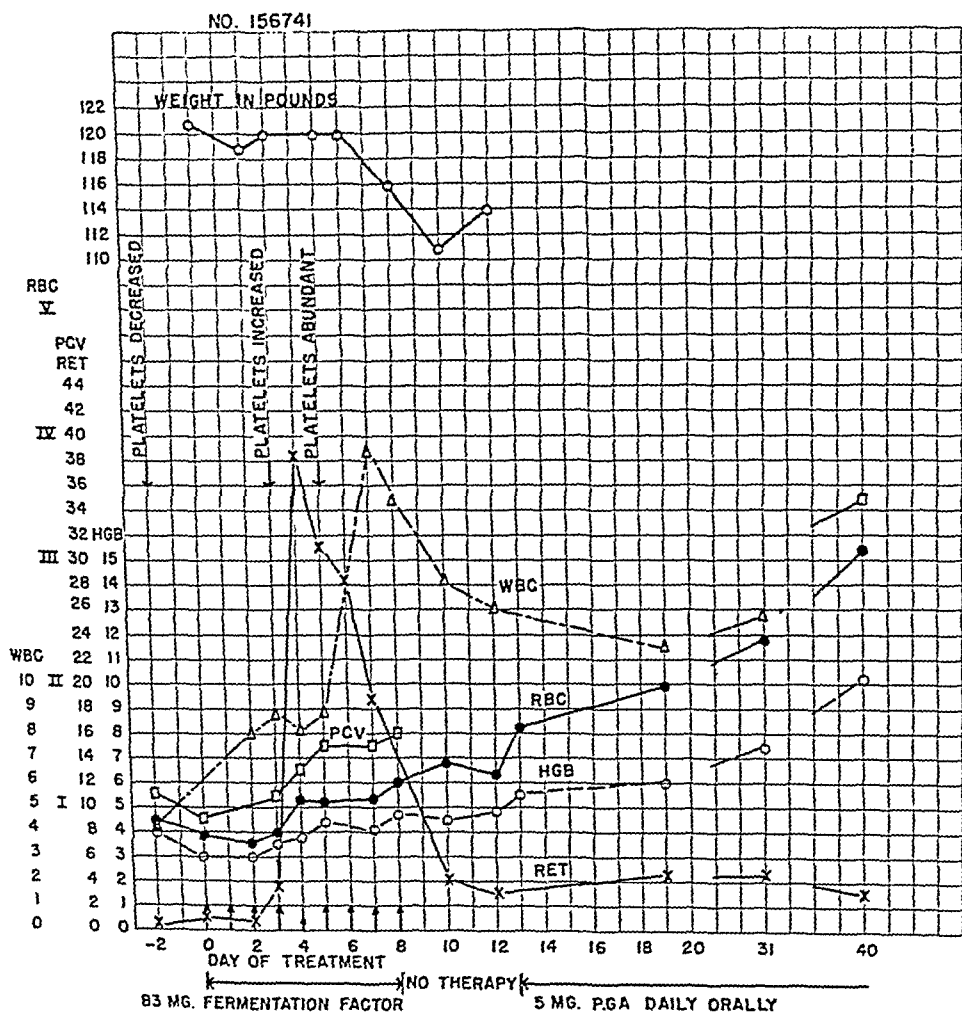


Fig. 1.—Hematologic response of patient with sprue following the intramuscular administration of fermentation factor. A total dose of 83 mg. was given during a nine-day period. Therapy was then discontinued for four days, following which it was resumed with 5 mg. daily of pteroylglutamic acid orally.

A diagnosis of sprue was made and on the second hospital day the patient was started on 5 mg. of fermentation factor intramuscularly twice daily. On the third day of therapy the reticulocyte count rose to 3.6 per cent, and on the fourth day a peak of 38.2 per cent was reached. The other hematologic data are presented in Fig. 1. On the second day of therapy the patient experienced a sense of well-being and considerable increase in appetite. On the fourth day beginning regeneration of the lingual papillae was noted. During the reticulocytosis the edema was pronounced, to subside within the next six days simultaneously with a decreased weight loss (Fig. 1). On the seventh hospital day the stools were reduced in number to one or two per day. On the seventh hospital day the patient complained of pain in the left leg associated with tenderness over the femoral triangle. This was associated with

increased edema of this extremity, but there were no changes in skin temperature, color, or sweating. Homan's sign was negative. A lumbar sympathetic novocain block provided no relief. The pain and edema gradually subsided with elevation of the extremities.

A total quantity of 83 mg. of the fermentation factor was administered during the nine days. No additional therapy was instituted until on the thirteenth day of therapy when the patient left the hospital under protest because she wished to return home. She was discharged on 5 mg. of PGA daily by mouth. Continued evidence of hemoregeneration has occurred.

From the beginning of her hospital stay the patient exhibited fever; the temperature ranged from 100 to 100.6° Fahrenheit. Penicillin was administered in doses of 25,000 units every three hours for ten days at the end of which time the temperature became normal.

#### DISCUSSION

This case met the criteria which we have employed in the diagnosis of sprue.<sup>10, 11, 12</sup> The patient gave no history of having received previous therapy with liver, and she was maintained during the hospital stay on a diet free of organ meats. The sole hemopoietic agent administered was the pteroyldiglutamylglutamic acid. While spontaneous remissions can occur in sprue, the character and timing of the hematologic changes and the simultaneous clinical improvement indicate convincingly that this remission was due to the administered fermentation factor. It is not valid to attempt to make a quantitative comparison of the response of this patient to the fermentation factor with the expected or standard response of other patients, inasmuch as the course was complicated by thrombophlebitis which may have reduced the height of the maximum reticulocyte response and also the rate of regeneration of red cells. The response exhibited by our patient demonstrates that a patient with sprue who had not been treated previously with liver extract has responded to the fermentation factor, thus supplying the conclusive evidence called for by Suárez and associates<sup>14</sup> of the activity of this substance in sprue without "the participation of exogenous factors of liver extract." This finding, together with the similar clear-cut response of a patient with nutritional macrocytic anemia reported by Goldsmith,<sup>15</sup> indicates that this member of the vitamin M group is hemopoietically active for man as well as for monkeys.

The activity of crystalline fermentation factor in sprue is additional evidence in favor of the hypothesis<sup>10, 11</sup> that vitamin M deficiency in the monkey is the experimental analogue of sprue in man. Since both sprue and nutritional macrocytic anemia respond to fermentation factor, this is a further link in the evidence for the similarity of these two syndromes.

#### SUMMARY

A patient with sprue was treated intramuscularly twice daily with 5 mg. of fermentation factor (pteroyldiglutamylglutamic acid). A total quantity of 83 mg. was given over a period of nine days. A maximum reticulocytosis of 38 per cent occurred on the fourth day of therapy. This was accompanied by a rise in erythrocyte count and hemoglobin and clinical improvement.

These results furnish additional evidence of the activity of this member of the vitamin M group in man and of the correctness of the hypothesis that the experimental analogue of sprue is vitamin M deficiency in the monkey.

## REFERENCES

1. Anonymous: Some Relations of Vitamin B<sub>12</sub> Conjugate, *Nutrition Rev.* 4: 15, 1946.
2. Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., Subbarow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr.: The Structure and Synthesis of the Liver L. casei Factor, *Science* 103: 667, 1946.
3. Stokstad, E. L. R.: Some Properties of a Growth Factor for *Lactobacillus casei*, *J. Biol. Chem.* 149: 573, 1943.
4. Hutchings, B. L., Stokstad, E. L. R., Bohonos, N., and Slobodkin, N. H.: Isolation of a New *Lactobacillus casei* Factor, *Science* 99: 371, 1944.
5. Piffner, J. J., Calkins, D. G., Bloom, E. S., and O'Dell, B. L.: On the Peptide Nature of Vitamin B<sub>12</sub> Conjugate From Yeast, *J. Am. Chem. Soc.* 68: 1392, 1946.
6. Piffner, J. J., Calkins, D. G., O'Dell, B. L., Bloom, E. S., Brown, R. A., Campbell, C. J., and Bird, O. D.: Isolation of an Antianemia Factor (Vitamin B<sub>12</sub> Conjugate) in Crystalline Form From Yeast, *Science* 102: 228, 1945.
7. Bethell, F. H., Swendseid, M. E., Bird, O. D., Meyers, M. C., Andrews, G. A., and Brown, R. A.: Observations on the Utilization of Folic Acid (Vitamin B<sub>12</sub>) and Vitamin B<sub>12</sub> Conjugate by Persons With Pernicious Anemia and Macrocytic Anemia Following Gastrectomy, *Univ. Hosp. Bull., Ann Arbor* 12: 42, 1946.
8. Welch, A. D., Heinle, R. W., Nelson, E. M., and Nelson, H. V.: Ineffective Utilization of Conjugated Pteroylglutamic (Folic) Acid in Pernicious Anemia, *J. Biol. Chem.* 164: 787, 1946.
9. Spies, T. D., and Stone, R. E.: Some Recent Experiences With Vitamins and Vitamin Deficiencies, *South. M. J.* 40: 46, 1947.
10. Darby, W. J., and Jones, E.: Treatment of Sprue With Synthetic L. casei Factor (Folic Acid, Vitamin M), *Proc. Soc. Exper. Biol. & Med.* 60: 259, 1945.
11. Darby, W. J., Jones, E., and Johnson, H. C.: The Use of Synthetic L. casei Factor in the Treatment of Sprue, *Science* 103: 108, 1946.
12. Cooperman, J. M., Elvehjem, C. A., McCall, K. B., and Ruegamer, W. R.: "Folic Acid" Active Compounds in the Nutrition of the Monkey, *Proc. Soc. Exper. Biol. & Med.* 61: 92, 1946.
13. Day, P. L., and Totter, J. R.: Studies on the Nutrition of the Monkey, With Special Reference to the *Lactobacillus casei* Factor (Vitamin M, Factor B<sub>12</sub>), Reported at the A.A.A.S. Conference on Vitamins, Gibson Island, July 31, 1946.
14. Suárez, R. M., Welch, A. D., Heinle, R. W., Suárez, R. M., Jr., and Nelson, E. M.: Effectiveness of Conjugated Forms of Folic Acid in the Treatment of Tropical Sprue, *J. LAB. & CLIN. MED.* 31: 1294, 1946.
15. Castle, W. B., Ross, J. B., Davidson, C. S., Burchenal, J. H., Fox, H. J., and Ham, T. H.: Extrinsic Factor in Pernicious Anemia: Ineffectiveness of Purified Casein and of Identified Components of the Vitamin B Complex, *Science* 100: 81, 1944.
16. Goldsmith, G. A.: The Treatment of Macrocytic Anemia With *Lactobacillus casei* Factor (Pteroylglutamic Acid), *J. LAB. & CLIN. MED.* 31: 1186, 1946.
17. Spies, T. D.: The Activity of Another L. casei Factor in Macrocytic Anemia, *South. M. J.* 39: 634, 1946.
18. Darby, W. J., Cherrington, M. E., and Ruffin, J. M.: Plasma Tocopherol Levels in Sprue, *Proc. Soc. Exper. Biol. & Med.* 63: 310, 1946.
19. Darby, W. J., Jones, E., and Johnson, H. C.: Effect of Synthetic *Lactobacillus casei* Factor in Treatment of Sprue, *J. A. M. A.* 130: 780, 1946.



## THIOURACIL AND PROPYLTHIOURACIL: A COMPARATIVE CLINICAL STUDY

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EXPERIMENTALLY, Astwood and Vander Laan<sup>1</sup> found propylthiouracil to be ten times more effective than thiouracil by weight as a goitrogenic agent in rats. Because of this increased effectiveness, propylthiouracil has been used in smaller doses<sup>2</sup> than thiouracil to produce the same change in thyroid physiology without incurring such a high incidence of toxic reactions to the drug. On the other hand, we have shared with Bartels<sup>3</sup> the clinical opinion that propylthiouracil in the doses used at present is not as effective as thiouracil. Before increasing the dosage of propylthiouracil in our thyrotoxic patients, we decided to analyze our results to date statistically to prove or disprove this clinical opinion. In general, the results of this analysis presented herein do not indicate that an increase in propylthiouracil dosage over 150 mg. per day is advantageous in the treatment of thyrotoxicosis.

### MATERIAL

Since March, 1946, we have used propylthiouracil as the initial form of treatment in forty-two thyrotoxic patients for a minimum period of six weeks. The distribution of patients treated with this drug, according to the number of months, is demonstrated in Table I.

Pertinent data necessary to compare the response rate of the two groups treated are presented in Table II. Other factors not listed in this table were identical in these two groups, since we directed the treatment in each group in the same manner but, starting in March, 1946, merely changed from thiouracil to propylthiouracil in the initial treatment of thyrotoxic patients. The general plan of treatment has been outlined in previous publications.<sup>4-7</sup>

Examination of Table II reveals major discrepancies at three points in these comparative data. In our opinion, the difference in the number of patients treated will not invalidate a comparison because (1) a statistical analysis of the first forty-seven patients treated with thiouracil<sup>4</sup> was not significantly different from the final analysis of the entire group of ninety-eight patients presented here and (2) we have continued the comparative graphs to be presented later in this paper only to the end of the first six weeks of treatment so that a maximum number of points would determine the position of the right end of the propylthiouracil line. The 25 per cent difference in the number of patients hospitalized

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TABLE I. DISTRIBUTION OF PATIENTS TREATED WITH PROPYLTHIOURACIL BY NUMBER OF MONTHS

MONTHS	PATIENTS
1 to 2	12
2 to 4	15
4 to 6	12
6 to 8	3

TABLE II. FACTORS AFFECTING COMPARISON OF THIOURACIL- AND PROPYLTHIOURACIL-TREATED GROUPS

	THIOURACIL	PROPYLTHIOURACIL
Number of patients	98	42
Per cent hospitalized initially	76	50
Average period of hospitalization	5.5 wk.	5.4 wk.
Average initial B.M.R. per cent	38	41
Average frequency of B.M.R.s		
Inpatients	1 per wk.	1 per wk.
Outpatients	1 per 1.5 mo.	1 per 1 mo.
Per cent of nodular goiters	39	40
Average dose	0.6 Gm.	0.150 Gm.

initially in each group is insignificant in view of the graph comparing the results in each hospitalized group. The marked difference in drug dosage then is the only significant variable between the two groups.

#### RESULTS

The following graphs (Figs. 1 to 3) summarize the results of statistical comparison of the group of ninety-eight patients treated with 0.6 Gm. of thiouracil per day and the forty-two patients treated with 0.15 Gm. of propylthiouracil per day.

Illustrated in Fig. 1 is the fact that the rate of descent of the basal metabolic rate in both groups of hospitalized patients is almost identical. In general, the clinical change in the patient corresponded to the change in the basal metabolism.

It is demonstrated in Fig. 2 that in patients treated with propylthiouracil the initial response of the thyrotoxic state to treatment accompanied by bed rest is greater than when the patient is ambulatory, although the eventual result is apparently the same. Since all outpatients in our series were urged to spend a minimum of twelve hours a day in bed, there might be even greater discrepancies if the outpatients' activities were not restricted. It should be noted also that in general we have hospitalized the more severely toxic patients as an initial precaution.

An equal rate of improvement in the thiouracil- and propylthiouracil-treated groups is shown in Fig. 3. This similarity of response, in spite of a clinical opinion to the contrary, is reminiscent of our early use of thiouracil.<sup>4</sup> Although we did not feel that 0.4 Gm. per day of thiouracil was the optimum dose, a statistical comparison of the patients treated on 0.4 and 0.6 Gm. per day later revealed no significant difference in therapeutic effect. It should be noted that the basal metabolic rate in thyrotoxic patients in Michigan does not fall at the rate of 1 per cent per day, as noted by Bartels<sup>5</sup> for patients on the Atlantic Coast, but at a rate of less than 1 per cent every two days.

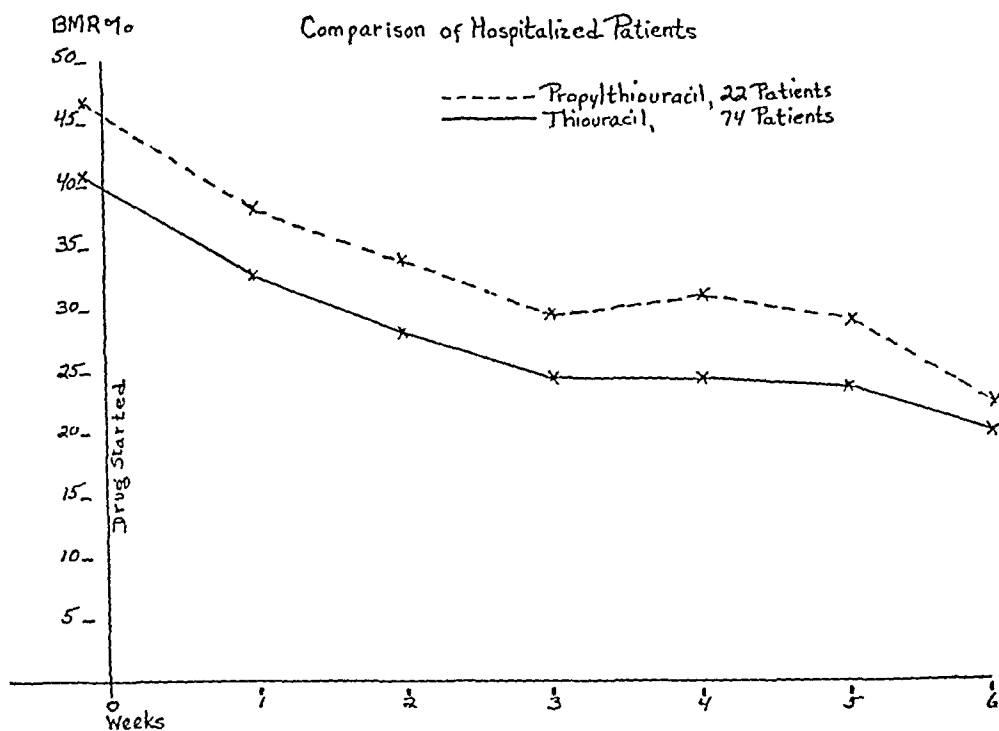


Fig. 1.

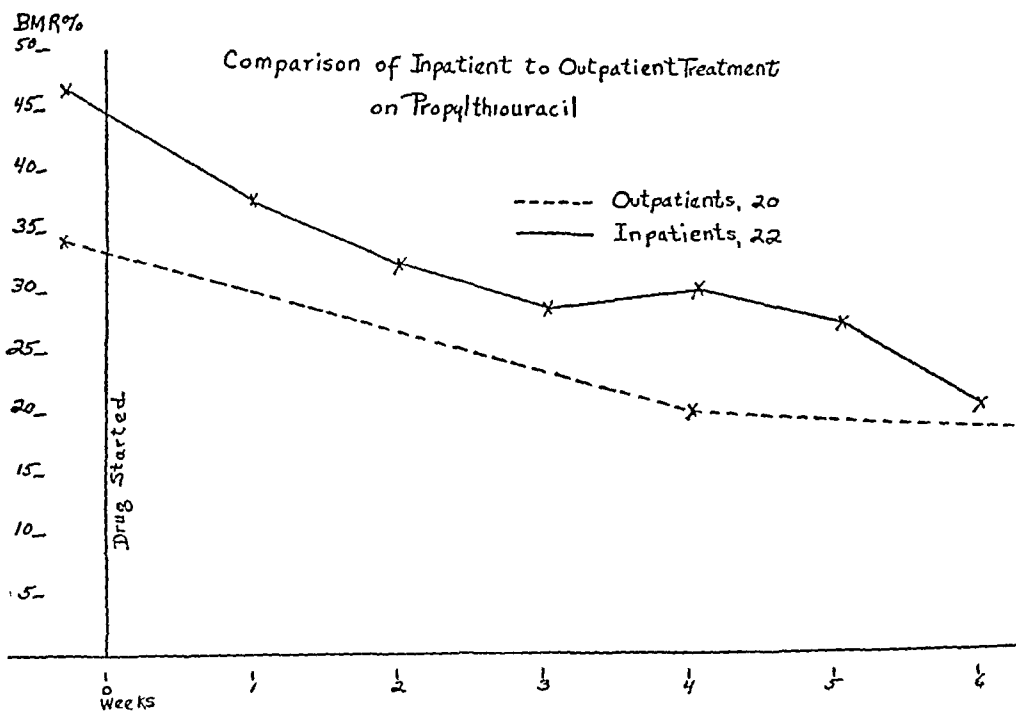


Fig. 2.

## DISCUSSION

This statistical comparison of the two drugs indicates that 150 mg. of propylthiouracil per day is an adequate dose for most thyrotoxic patients. The only toxic reactions we have observed in nine months of use of this new anti-thyroid agent are (1) drowsiness during the first two weeks of administration in about 30 per cent of the patients and (2) acneform dermatitis of the face in one patient, which disappeared when the dosage of the drug was reduced by 50 mg. per day. The lack of toxic reactions with this agent in our series is in keeping with the observation of Astwood and Vander Laan.<sup>2</sup> However, Bartels<sup>3</sup> mentioned three cases of agranulocytosis presumably due to propylthiouracil. Since propylthiouracil lacks toxicity mainly by virtue of its decreased dosage, the occurrence of the more severe forms of toxic reactions such as agranulocytosis and drug fever might be logically expected if the amount of drug administered is increased. Fortunately, our experience indicates that an increase in dosage is not necessary to achieve results comparable to those with thiouracil.

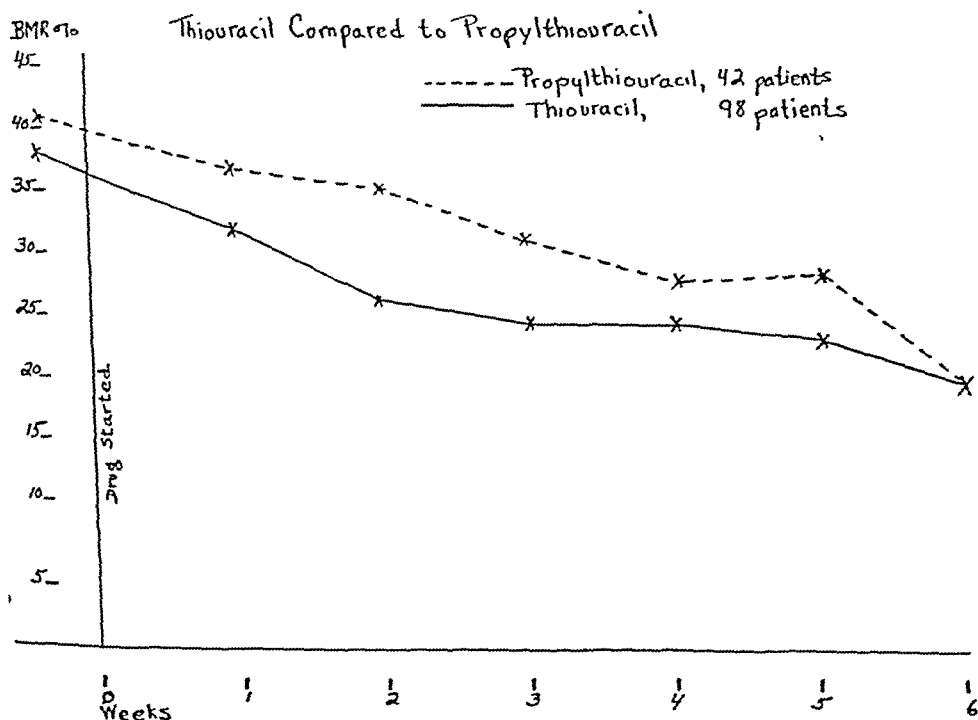


Fig. 3.

## SUMMARY

1. A statistical comparison of ninety-eight thyrotoxic patients treated with 0.6 Gm. of thiouracil per day and forty-two treated with 0.15 Gm. of propylthiouracil per day reveals practically identical effectiveness of the two drugs in these doses.

2. An initial period of bed rest coupled with the administration of propylthiouracil is accompanied by a greater favorable initial response than in the ambulatory-treated patient, although the end result appears to be the same.

3. In Michigan the average rate of response of the basal metabolic rate of thyrotoxic patients treated with either drug is less than a 1 per cent fall every two days, in contrast to a fall twice as great reported from the Atlantic Coast.

4. The incidence of toxic reactions with propylthiouracil is much less than with thiouracil.

5. Severe toxic reactions well might be encountered if the present dosage of propylthiouracil is significantly increased.

6. The available data indicate that 150 mg. is probably the most satisfactory maximum dosage of propylthiouracil.

We are indebted to the staff of the Department of Internal Medicine and to Dr. F. A. Coller and Dr. R. W. Buxton of the Department of Surgery for their cooperation in making the patients available to us for study.

#### REFERENCES

1. Astwood, E. B., and Vander Laan, W. P.: Thiouracil Derivatives of Greater Activity for the Treatment of Hyperthyroidism, *J. Clin. Endocrinol.* 5: 424, 1945.
2. Astwood, E. B., and Vander Laan, W. P.: Treatment of Hyperthyroidism With Propylthiouracil, *Ann. Int. Med.* 25: 83, 1946.
3. Bartels, E. C.: Personal Communication at the American Goiter Association Meeting, Chicago, Ill., June, 1946.
4. Beierwaltes, W. H.: Forty-seven Patients Treated With Thiouracil, *Univ. Hosp. Bull., Ann Arbor* 11: 102, 1945.
5. Beierwaltes, W. H., and Sturgis, C. C.: Remissions in Thyrotoxicosis After Discontinuing Thiouracil, *J. A. M. A.* 131: 735, 1946.
6. Beierwaltes, W. H., and Sturgis, C. C.: Complications Following the Administration of Thiouracil, *Am. J. M. Sc.* 212: 513, 1946.
7. Beierwaltes, W. H.: Remissions in Thyrotoxicosis After Thiouracil, *Tr. Am. Goiter Ass.*, June, 1946. To be published.
8. Bartels, E. C.: Use of thiouracil in Preoperative Preparation of Patients With Severe Hyperthyroidism, *Ann. Int. Med.* 22: 365, 1945.

# OBSERVATIONS ON THE DIURNAL EXCRETION OF UROBILINOGEN IN THE URINE OF NORMAL SUBJECTS AND OF PATIENTS WITH LAËNNEC'S CIRRHOSIS

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THERE have been many reports on the diurnal variation of urobilin or urobilinogen excretion in normal and pathologic states. These studies have been performed with techniques differing in sensitivity and reliability and in the main on small groups of cases. In 1893 Grimm<sup>1</sup> and in 1897 Sallet<sup>2</sup> independently showed (1) that the excretion of urobilin during the day exhibits two peaks, one in midmorning and the other in midafternoon and (2) that nocturnal excretion of this substance constitutes a very small portion of the twenty-four hour excretion. Subsequent reports<sup>3-10</sup> are in general agreement that the maximal urobilin or urobilinogen excretion takes place in the afternoon and that minimal excretion takes place at night. Variations have been attributed to digestive and metabolic activity.

Wallace and Diamond,<sup>10</sup> for example, tested the urobilinogen excretion in morning and afternoon urines of 125 medical students. Their method depends upon the red color reaction produced by Ehrlich's aldehyde reagent with urobilinogen. A rough estimate of urobilinogen concentration is made by diluting the urine until the pink color disappears, a dilution of 1:20 being considered the outer limit of normal. Fifty specimens obtained in the afternoon showed a slightly higher concentration of urobilinogen than seventy-five specimens obtained in the morning. White and associates,<sup>8</sup> employing the Wallace and Diamond method, tested for urobilinogen at two-hour intervals during the day in fifteen normal subjects. "The variation in the results was found to be moderate and all within normal limits. . . . The afternoon specimens showed more urobilinogen than the morning specimens in about one-half of the cases." Steigmann and Dyniewicz<sup>9</sup> determined the hourly excretion of three normal subjects over a twenty-four hour period. In one subject the maximal excretion was at 4 P.M. and in one at 8 P.M., whereas the third subject showed peaks at 10 A.M. and 4 P.M. In comparing the morning and afternoon urine samples of twelve patients with urobilinogenuria from various diseases, Watson and co-workers<sup>11</sup> found the concentration of the afternoon specimens to be higher in nine of twelve instances.

Most of the preceding reports are concerned with the relative concentrations of urobilinogen excreted in morning and afternoon urine samples. It seemed desirable to measure the quantity excreted per unit time as well, since a highly concentrated urine might give a false impression of the total excretion. With

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the introduction by Watson and co-workers<sup>11</sup> of a simplified method for the quantitative determination of urobilinogen, it became feasible to re-examine the problem of diurnal excretion of urobilinogen in a series of patients with liver disease. It was of practical interest to ascertain (1) whether there is an optimal period for urobilinogen excretion and (2) whether the daily pattern of excretion is constant in the same patient and in different patients. With this purpose the urobilinogen excretion was determined at three periods of the day in a series of normal subjects and in patients with cirrhosis of the liver.

#### METHOD

The method employed is essentially that described by Watson and associates<sup>11</sup> in which the Ehrlich color reaction is measured in a photoelectric colorimeter. In our procedure a few minor changes were introduced. Instead of a blank composed of reagents added in reverse order, as employed by these authors, we have employed a blank of 22 per cent HCl, which is the concentration of HCl contained in the Ehrlich reagent itself. The procedure is as follows: unknown, 2.5 c.c. urine, 2.5 c.c. "Ehrlich Reagent,"\* 5 c.c. saturated sodium acetate; blank, 2.5 c.c. urine, 2.5 c.c. 22 per cent HCl,† 5 c.c. saturated sodium acetate.

It seemed essential to standardize the procedure with reference to time and temperature, since these factors influence the intensity of color reaction. The first two reagents are mixed and placed in a water bath at 37° C. for ten minutes. Colorimetry then is performed directly after the addition of the sodium acetate solution. The color obtained in this manner is generally maximal for the reaction.

Readings were made with the Klett photoelectric colorimeter with a No. 56 filter‡ and calibrated against the Pontacyl violet stock solution described by Watson and associates.<sup>11</sup>

Results are expressed in terms of Ehrlich units per hour (as proposed by Watson), since the reaction is not specific for urobilinogen but measures other Ehrlich-reacting substances as well. These chromogens are said to parallel roughly the excretion of urobilinogen.

The subjects of this study were thirty-six hospitalized patients with cirrhosis of the liver observed during a period of two years on the Research Service (First Division) of the Goldwater Memorial Hospital. The diagnosis was established in each case by history, physical findings, and laboratory tests of liver function. All but four cases had the diagnosis confirmed either by biopsy of the liver or by necropsy. Twenty cases were classified as in severe failure and sixteen in moderate failure. All patients were being treated according to a dietary regimen previously described.<sup>12</sup> This consisted of a nutritious diet, rich in protein and supplemented with 50 Gm. of brewers' yeast daily. No fluid or dietary restrictions were imposed preceding or during the test.

The patients voided at 7 A.M. Urine excreted from 7 to 10 A.M., 10 A.M. to 1 P.M., and 1 to 4 P.M. was then collected in separate bottles. Determinations were performed on each three-hour specimen within fifteen minutes of its collection. In this manner 180 series of determinations or a total of 540 single determinations were performed on thirty-six patients. The distribution of tests in this group was as follows: thirteen patients had one serial determination; five had three or four determinations; ten had five or more; and eight had ten or more.

The control series consisted of thirty-nine healthy subjects who were medical students and hospital personnel ranging in age from 20 to 35 years. Fifty-seven tridaily determinations or a total of 171 single determinations were made. The distribution of tests in this group was as follows: twenty-seven subjects had one serial determination; seven had two determinations; four had three; and one subject had four.

\*0.7 Gm. *p*-dimethylaminobenzaldehyde, 150 c.c. concentrated HCl, 100 c.c. distilled H<sub>2</sub>O.

†150 c.c. concentrated HCl, 100 c.c. distilled H<sub>2</sub>O.

‡There is little difference between values obtained with a No. 54 filter and those with a No. 56 filter. The procedure also has been carried out with a Junior Coleman colorimeter at 565 millimicrons.

## RESULTS

The periods of maximal urine urobilinogen excretion both in normal subjects and in patients with liver cirrhosis are shown in Table I. There were fifty-seven serial determinations in normal subjects, of which sixteen were highest in the morning, nineteen at midday, nineteen in the afternoon, and three showed no peak of excretion. In patients with cirrhosis (180 serial tests), sixty-four showed maximal excretion in the morning, fifty-two at midday, and sixty-four in the afternoon.

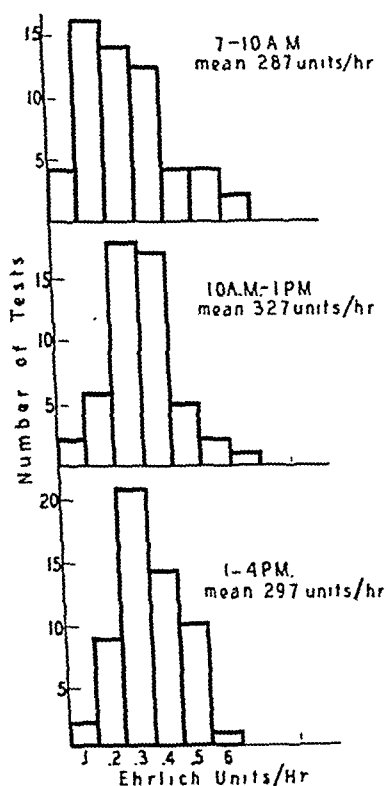


Fig. 1.

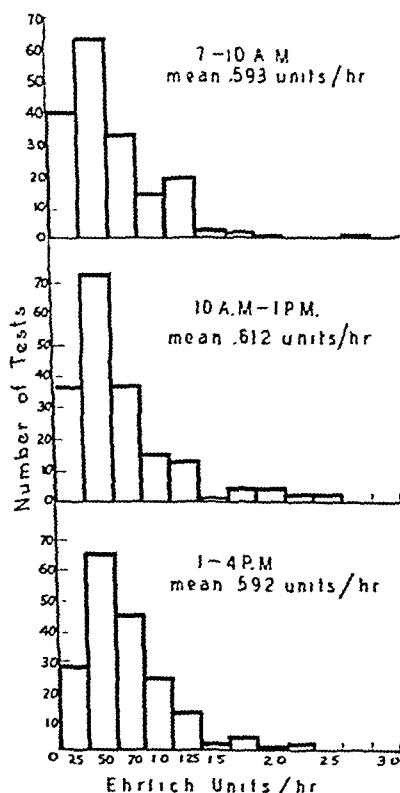


Fig. 2.

Fig. 1.—Diurnal urine urobilinogen excretion in 39 normal subjects. Mean values are similar for the three periods of the day. Few determinations exceed 0.5 unit per hour.

Fig. 2.—Diurnal urine urobilinogen excretion in 36 patients with liver cirrhosis. The pattern is similar to that of the normal subjects. Because of a wider distribution of values the scale has been reduced in this illustration.

The data are presented graphically in Figs. 1 and 2. Each histogram shows the number of determinations falling within a given range of urobilinogen excretion in units per hour. The distribution of these values does not differ significantly during the three periods, either for the normal subjects or the patient group. The similarity in the form of the graphs for the three periods suggests that no peculiar trend of urobilinogen excretion takes place during the day.



TABLE I. PERIODS OF MAXIMAL UROBILINOGEN EXCRETION

TIME	NORMAL SUBJECTS		PATIENTS WITH LIVER CIRRHOSIS	
	NUMBER OF INSTANCES	PER CENT	NUMBER OF INSTANCES	PER CENT
7 to 10 A.M.	16	29.6	64	35.5
10 A.M. to 1 P.M.	19	35.2	52	29.0
1 to 4 P.M.	19	35.2	64	35.5

The mean value of the normal subjects was 0.3 unit per hour. In 95 per cent of the tests made on normal subjects, values of 0.5 unit or less per hour were obtained. This finding is in agreement with the normal range obtained by Watson and co-workers.<sup>11</sup> In the case of liver cirrhosis the mean urinary excretion was 0.6 unit per hour. There was a much wider spread of values in this patient group, that is, from 0.03 to 5.4 units per hour. *Although all patients with cirrhosis of the liver showed increased urobilinogen excretion at one or another time, there were many tests that fell within the normal range.* In fact, fifty-five per cent of 540 tests on these patients fell within the normal range. The need for repeated tests becomes obvious.

The diurnal urobilinogen excretion of eight patients who had ten or more serial determinations made is shown in Table II. In each case the diagnosis of liver cirrhosis was confirmed by biopsy. Two points are illustrated in Table II: first, that while some patients favor one period for maximal excretion others do not; second, that the pattern of urobilinogen excretion may vary in the same patient at different times.

In general, there was a poor correlation between the severity of liver disease and the degree of urobilinogen excretion (Table II). The poor correlation stands out in contrast to the experience of others with acute hepatitis in which there appears to be close agreement between urobilinogen excretion and the activity of the disease process. However, when jaundice was present in patients with cirrhosis consistently higher values were obtained. This has been observed previously by Steigmann and Dyniewicz.<sup>12</sup>

Morning and afternoon peaks of excretion were noted in only a few instances. The peaks described by Grimm,<sup>1</sup> Sallet,<sup>2</sup> and Bang<sup>5</sup> were transitory and took place two or three hours after meals. Since the present study involves consecutive three-hour periods, those sharp variations might have been neutralized by the long periods of collection.

Salen,<sup>4</sup> Bang,<sup>5</sup> and White and associates<sup>8</sup> found no relation between the volume of urine and the amount of urobilinogen excreted. Our data tend to confirm their findings. However, it appears that urobilinogen excretion can be increased by excessive intake of water. This effect was tested in two normal subjects. After ingestion of 1,500 c.c. of water one subject showed little, if any, increase in urobilinogen excretion on two tests. In another subject the urobilinogen excretion after the ingestion of 1,500 and 5,000 c.c., respectively, was two and three times greater than that of the preceding control periods.

TABLE II. SUCCESSIVE TESTS OF URINE UROBILINOGEN EXCRETION IN EIGHT PATIENTS WITH LIVER CIRRHOSIS

PATIENT	CLINICAL SEVERITY	TIME	UNITS PER HOUR												
K.	4+	7 to 10 A.M.	1.02	1.44	1.10	5.10	1.20	1.13	.41	.24	.65	.88	.83	.32	1.17
		10 A.M. to 1 P.M. 1 to 4 P.M.	.54 .51	.62 1.02	1.01 1.15	1.15 1.71	.71 .79	.94 .54	.34 .98	1.23 .48	1.00 1.24	.92 .32	1.70 1.96		
M. D.	2+	7 to 10 A.M.	.48	.24	.26	.27	.51	.21	.40	.13	.73	1.24	.22	.46	
		10 A.M. to 1 P.M. 1 to 4 P.M.	.28 .27	.63 .22	.28 .26	.27 .35	.20 .35	.28 .15	.28 .27	.32 .19	.41 .44	.27 .32	.35 .28	.45 .56	
R. F.	4+	7 to 10 A.M.	.16	.16	.39	.41	.22	.24	.25	.29	.13	.03	.13	.09	
		10 A.M. to 1 P.M. 1 to 4 P.M.	.05 .13	.58 .06	.73 .25	.42 .63	.62 .50	.24 .60	.32 .44	.33 .31	.05 .06	.18 .26	.05 .19		
T. S.	1+	7 to 10 A.M.	.54	.80	.42	.73	.64	.89	.81	.39	.57	.75	.60	.43	1.13
		10 A.M. to 1 P.M. 1 to 4 P.M.	.43 .57	.69 .68	.45 .83	.52 .99	.32 .75	.40 .68	.79 .63	.38 .58	.57 .60	.68 .55	.54 .66	.96 .59	1.12 .76
H.	4+	7 to 10 A.M.	.52	.28	.39	.42	1.25	.53	.13	.28	.70	1.16	.92	1.51	.89
		10 A.M. to 1 P.M. 1 to 4 P.M.	.49 .32	.29 .56	.38 .60	.57 .33	.69 .31	.30 .45	.38 .27	.27 .26	.19 .35	.53 .88	.20 .32	1.12 1.10	.89
C. G.	4+	7 to 10 A.M.	.67	.81	.32	.60	.79	2.63	.15	.25	.11	1.10	.80	.92	1.04
		10 A.M. to 1 P.M. 1 to 4 P.M.	.31 .46	1.05 1.07	.44 .30	.43 .35	.88 .61	1.43 1.63	.64 1.27	.05 .21	1.15 1.59	1.68 1.74	1.21 .75	1.82 2.34	.51 1.10
W. M.	2+	7 to 10 A.M.	.44	.37	.66	.41	.61	.43	.53	.85	.18	.33	.61	1.42	.47
		10 A.M. to 1 P.M. 1 to 4 P.M.	.47 .41	.36 .08	.46 .64	.48 .54	.64 .52	.60 .65	.61 .23	.49 .45	.13 .41	.25 .32	.70 .52	.89 .87	.34 .30
M. D.	3+	7 to 10 A.M.	.75	.52	.54	.47	.50	.31	.50	.30	1.05	.66	.29	.55	.22
		10 A.M. to 1 P.M. 1 to 4 P.M.	.40 .93	.48 .19	.31 .40	.35 .24	.48 .62	.30 .53	.49 .33	.41 .43	.30 .32	.69 .25	.25 .26	.60 .53	.48 .30

## SUMMARY AND CONCLUSIONS

1. Urine urobilinogen excretion was determined for three successive periods of three hours each in thirty-nine normal subjects and in thirty-six patients with cirrhosis of the liver.

2. The mean urine urobilinogen excretion per hour was 0.3 Ehrlich unit for the normal subjects and 0.6 for the patients.

3. The period of maximal urobilinogen excretion varied in the different subjects and also in the same subject at different times.

4. There was a poor correlation between the degree of urobilinogenuria and the clinical severity of the liver disease.

## REFERENCES

1. Grimm, F.: Ueber Urobiline im Harn. *Virchows Arch. f. path. anat.* 132: 246, 1893.
2. Salliet: De l'urobiline dans les urines normales, *Rev. de med.* 17: 109, 1897.
3. Wilbur, R. L., and Addis, T.: Urobilin: Its Clinical Significance, *Arch. Int. Med.* 13: 236, 1914.
4. Salen, E. B.: Urobilinstudien, *Acta med. Scandinav.* 60: 291, 1924.
5. Bang, O.: Klinische Urobilinstudien in Sonderheit an normalen und "lebergesunden" Personen, *Acta med. Scandinav. Supp.* 29.
6. Watson, C. J.: Urobilinogen in Urine and Feces of Subjects Without Evidence of Disease of Liver or Biliary Tract, *Arch. Int. Med.* 59: 196, 1937.
7. Watson, C. J.: The Per Diem Excretion of Urobilinogen in the Common Forms of Jaundice and Disease of the Liver, *Arch. Int. Med.* 59: 206, 1937.
8. White, F. W., Meiklejohn, A. P., Deutsch, E., Kark, R.: A Comparison of 3 Urobilinogen Tests in the Urine (the Watson, Sparkman, and Wallace and Diamond Methods), in *Jaundice and Diseases of the Liver*, *Am. J. Digest. Dis.* 8: 346, 1941.
9. Steigmann, F., and Dyniewicz, J. M.: Studies of Urobilinogen. I. The Daily Excretion in Urine and Feces in Health and Disease; an Evaluation of the Watson's and Sparkman's Methods, *Gastroenterology* 1: 743, 1943.
10. Wallace, G. B., and Diamond, J. S.: The Significance of Urobilinogen in the Urine as a Test of Liver Function, *Arch. Int. Med.* 35: 698, 1925.
11. Watson, C. J., Schwartz, S., Sborov, V., and Bertie, E.: Studies of Urobilinogen. V. A Simple Method for the Quantitative Recording of the Ehrlich Reaction as Carried Out With Urine and Feces, *Am. J. Clin. Path.* 14: 605, 1944.
12. Patek, A. J., Jr., and Post, J.: Treatment of Cirrhosis of the Liver by a Nutritious Diet and Supplements Rich in Vitamin B Complex, *J. Clin. Investigation* 20: 481, 1941.
13. Steigmann, F., and Dyniewicz, J. M.: Studies of Urobilinogen. II. Quantitative Urobilinogen Determinations in the Differential Diagnosis of Jaundice, *Gastroenterology* 1: 855, 1943.

# A COMPARISON OF THE NUTRITIVE VALUE OF THE PROTEINS IN MIXED DIETS FOR DOGS, RATS, AND HUMAN BEINGS

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THE evaluation of the nutritive value of proteins has usually been done with rats, although some data have been collected using other species such as the canine, man, and the avian. In general, it is known that the supplementation of vegetable diets with a high quality protein such as meat causes an improvement in the nutritive value of the ration for all species which have been studied. The essential amino acids for the rat, human being, dog, and chick are similar but not the same. It seems likely that the quantitative relationships of the essential amino acids may also vary among the species. Such differences, should they occur, could only mean that various proteins would have different biologic values for different species. The relatively poor nutritive value of casein for the chick due to its low arginine and glycine content is a specific example, but whether such differences occur among the other common species of laboratory animals is unknown.

Biologic value, usually defined as the per cent of absorbed nitrogen retained in the body, depends upon the amino acid composition of the absorbed protein. There may also be species differences in the digestibility of proteins. Although the reasons for differences in the digestibility of proteins are obscure, specific amino acid linkages, proteinase inhibitors, amount of fiber in the food, method of preparation of the food or protein, and amount and kind of the various proteinases in the digestive apparatus are factors which may be involved. There is little reason to believe that all factors would be of equal importance in different species. A predominantly carnivorous animal with a short digestive tract might be expected to digest the proteins in vegetable matter less effectively than predominantly vegetarian species.

With these theoretic possibilities in mind, several diets which had previously been studied with human subjects<sup>1</sup> were fed to rats and dogs. In this paper the figures obtained for the digestibility and biologic values in these species are compared to those previously reported for man.

## EXPERIMENTAL

The diets which were examined as protein sources have been previously described.<sup>1</sup> The all-vegetable diet called Diet IB contained no animal protein. Approximately 50 per cent of the protein in this diet came from white bread made without milk solids; other percentages were: 7.6 from corn meal, 4.4 from rice, 13.3 from potatoes, 16.3 from other vegetables (including lettuce, carrots, onions, and tomatoes), and 8.4 from fruits (including orange juice, apple sauce, and peaches).

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When supplements were added, each constituent of Diet IB was decreased by one-third and the amount of protein thus removed was replaced by an equivalent amount of protein from the supplement. In this paper the diets are identified according to the supplement used, that is, meat, soy flour, wheat germ, or bread.

During the studies on human subjects<sup>1</sup> additional meals were prepared and dried at 40° C. in front of fans. After drying, the mixed diets were ground, analyzed for protein by the Kjeldahl method and for fat by ether extraction, and stored in the refrigerator until used in the present studies.

*Studies With Dogs:* Ten dogs were used in these studies. They varied in weight from 8 to 15 kilograms and were free from parasites. At the beginning of each study they were fed a diet essentially free of nitrogen of the following percentage composition: sucrose, 83; cottonseed oil, 9; salt mixture,<sup>2</sup> 4; and cod-liver oil, 2. Crystalline vitamins<sup>3</sup> were added to supply 200  $\gamma$  thiamine chloride, 400  $\gamma$  riboflavin, 200  $\gamma$  pyridoxine, 1.5 mg. pantothenic acid, 2.5 mg. nicotinic acid, and 100 mg. choline per 100 Gm. of diet. The diet was fed in amounts to supply approximately 80 calories per kilogram of body weight. When the protein supplements were fed, the amount of the nitrogen-free diet was decreased proportionately to keep the caloric intake approximately constant.

In general, the plan followed was to feed the nitrogen-free diet for a period of ten to twenty days. The urine and feces were collected during the last week. The urine was combined to give composite samples representing a three- and a four-day period. The feces were homogenized in a Waring blender with water and combined in a similar manner. Charcoal markers were used to delimit the feces into appropriate periods. Following the nitrogen-free period, supplements of the experimental diets were given. Generally supplements of the all-vegetable Diet IB were fed for one week followed by a week during which the diet containing meat was used to supply a similar amount of nitrogen. During the next week another level of Diet IB was fed and following this the meat diet at a similar level, etc. However, in some animals only Diet IB was fed, the levels being increased each week. The amount of supplement which was given varied from 0.50 to 1.80 Gm. of nitrogen per dog per day.

Considerable difficulty was met in completing satisfactory studies. The diets were apparently not palatable for dogs, at least after a depletion period, and many of the dogs went off feed within a few days after the supplement was given and had to be discontinued. Thus, more data were collected on the all-vegetable Diet IB than on the diet containing meat, and studies on wheat germ and soy flour supplemented diets were not attempted with dogs. Studies similar to those reported by Allison and Anderson<sup>3</sup> were unsuccessful for similar reasons, since the higher levels of the diets were even more difficult to feed.

*Studies With Rats:* These studies were made with adult male rats. They were fed a nitrogen-free diet of the following percentage composition: sucrose, 84; corn oil, 10; salts, 4; cod-liver oil, 2; and vitamins at the same levels

\*Supplied by Merck & Company, Inc., Rahway, N. J.

used in the diets for dogs. When supplements were added, these replaced sucrose and sufficient corn oil to maintain the fat content at 10 per cent. All supplemented diets contained 7.5 per cent protein ( $N \times 6.25$ ).

The experimental procedure was to feed the animals a nitrogen-free diet for twenty days, collections being made during the last eleven days in three periods of four, three, and four days each, respectively. Following this the diet containing protein from the all-vegetable diet was fed for twelve days, collections being made the last eleven days in four, three, and four day periods, respectively, as before. Then a similar twelve-day period followed during which one of the other diets was tested. The food intake during the nitrogen-free period was recorded and a like amount fed during the other periods. By this procedure each supplemented diet was compared to the all-vegetable diet. Twelve rats were used for each experiment.

Biologic values and digestibilities were calculated in the conventional manner. Digestibility is the per cent of fed protein absorbed and biologic value the per cent of absorbed nitrogen retained.

Studies also were made using young growing male rats (average weight 45 grams). In one experiment groups of six rats each were fed ad libitum on diets containing 7.5 per cent protein of a similar composition to those used for adult rats. In a second experiment using the same number of animals, each six animals of comparable weight receiving the different diets were restricted in food intake to the amount eaten by the animal having the lowest intake. This was usually the animal receiving the unsupplemented diet, but in some instances the bread-supplemented diet produced the least gain and food intake. In this study all the diets contained 9.6 per cent protein.

### RESULTS

The results obtained with dogs are shown in Table I. The average results indicate an increase in digestibility and of biologic value of approximately 10 per cent by the inclusion of meat in the diet. The standard deviations are large for each value, especially of those for biologic value. It is our impression that there is a great variation in the ability of dogs to "handle" diets such as those which are high in vegetable matter.

TABLE I. DIGESTIBILITY AND BIOLOGIC VALUE OF DIETS FOR ADULT DOGS

SUPPLEMENT TO DIET IB	DIGESTIBILITY		BIOLOGIC VALUE	
	NUMBER OF TESTS	AVERAGE $\pm$ STANDARD DEVIATION	NUMBER OF TESTS	AVERAGE $\pm$ STANDARD DEVIATION
None	27	80.0 $\pm$ 7.7	16	67 $\pm$ 21
Meat	11	90.3 $\pm$ 7.1	11	77 $\pm$ 14

The data from the adult rats are shown in Table II. These data were treated somewhat differently than those from the dogs, since for each rat data were available on the all-vegetable diet for comparison with the supplemented diet. The values obtained for Diet IB in each experiment involving twelve rats are recorded, and the significance of the difference between this value and the observed value for the supplemented diet is given. It may be noticed

TABLE II. DIGESTIBILITY AND BIOLOGIC VALUE FOR ADULT RATS OF SUPPLEMENTED DIETS COMPARED TO ALL-VEGETABLE DIET

SUPPLEMENT	DIGESTIBILITY				BIOLOGIC VALUE				NET VALUE		
	DIET IB	SUPPLEMENT	DIF-FERENCE	PROBABILITY OF SIGNIFICANCE	DIET IB	SUPPLEMENT	DIF-FERENCE	PROBABILITY OF SIGNIFICANCE	DIET IB	SUPPLEMENT	DIF-FERENCE
Meat	75.9	79.7	+ 3.8	0.15	68.1	80.7	+12.6	0.01	51.6	64.0	+12.4
Soy flour	79.1	91.4	+12.3	0.01	72.7	77.0	+ 4.3	0.10	57.4	70.2	+12.8
Wheat germ	83.3	89.2	+ 5.9	0.05	73.2	77.7	+ 4.5	0.03	61.0	69.1	+ 9.1
Average	79.4				71.3						

that whereas all three supplements allowed some improvement in both biologic value and digestibility, the increase in digestibility due to the addition of meat and the increase in biologic value from soy flour supplementation were not significant at the 5 per cent level.

The results with young growing rats are presented in Table III. In these studies all three supplements clearly improved the nutritive value of the diet, whether the diets were fed ad libitum or restricted to the intake of the poorest animal. In neither experiment are meat, wheat germ, or soy flour supplements significantly different. In these studies the diet containing additional bread studied with human beings<sup>1</sup> was also fed. A diet containing skim milk powder as the sole source of protein was included for comparison in this experiment. Skim milk powder appeared only slightly better than the meat-supplemented diet, while bread did not improve or decrease the nutritive value of the diet. The efficiency of protein utilization is not presented since, as has been shown,<sup>4</sup> this is a function of the weight of the animals.

TABLE III. WEIGHT GAIN IN YOUNG RATS RECEIVING VARIOUS DIETS

SUPPLEMENT TO DIET IB	AD LIBITUM FEEDING† (GM.)	RESTRICTED INTAKE‡ (GM.)
None	16.6 ± 7.0	8.0
Meat	61.0 ± 9.0	21.5
Wheat germ	57.0 ± 13.0	24.5
Soy flour	54.2 ± 16.3	22.5
Bread	17.5 ± 7.0	11.0
Skim milk powder*	73.0 ± 16.5	

\*Skim milk powder as the sole source of protein, not as a supplement to Diet IB.

†During fifty days on diets containing 7.5 per cent protein.

‡During twenty-one days on diets containing 9.6 per cent protein.

In additional experiments groups of animals receiving the basal all-vegetable and the meat-supplemented diets were placed in activity cages. No significant differences in spontaneous activity were observed either with restricted or ad libitum feeding.

#### DISCUSSION

In the previous publication<sup>1</sup> the digestibility and biologic value of the protein in the various diets for human beings were estimated, although the excretions on a nitrogen-free diet were not actually determined. The fecal nitrogen on a nitrogen-free diet was estimated by feeding the all-vegetable diet at

three different levels and extending the regression line to zero protein intake. The urinary excretion on a nitrogen-free diet was estimated to be 2 mg. of nitrogen per basal calorie. The reliability and criticisms of these methods have been discussed,<sup>5</sup> but it is believed that they are useful for comparative purposes.

The present results have been summarized with those obtained on human subjects in Table IV. In general, it may be said that the results on rats and dogs are similar to those obtained with human beings although there are some apparent differences. Our most reliable data on all species were obtained with the all-vegetable diet, and it appears that digestibility of this diet was relatively better in man than in the other two species. The biologic values, on the other hand, are in the same range for all species, although somewhat lower for dogs. This may be some evidence for the correctness of the assumed figure for the endogenous nitrogen excretion used in the calculations on the data for man. The net protein values suggest that man utilized the all-vegetable diet somewhat better than the other two species, because of more efficient digestion. This result, however, may be due to the methods used in determining the metabolic fecal nitrogen. Bosshardt and Barnes<sup>6</sup> have shown that fecal nitrogen of mice receiving a nitrogen-free diet underestimates the metabolic fecal nitrogen occurring when proteins are fed. These authors believe that the method used in these studies on human beings is preferable to feeding nitrogen-free diets. If this is true, the digestibility of the proteins in rats and dogs has been underestimated. These studies appear to confirm the observations of Bosshardt and Barnes.

TABLE IV. COMPARISON OF NUTRITIVE VALUE OF PROTEINS OF VARIOUS DIETS IN MAN, RATS, AND DOGS

SUPPLEMENT	ADULT MAN			ADULT RATS			ADULT DOGS			YOUNG RATS	
	DIGESTIBILITY	BIOLOGIC VALUE	NET	DIGESTIBILITY	BIOLOGIC VALUE	NET	DIGESTIBILITY	BIOLOGIC VALUE	NET	GAIN IN THREE WEEKS	
										AD LIBITUM (GM.)	LIMITED INTAKE (GM.)
None	87.5	72.5	63.5	79.4	71.3	56.5	80.0	66.7	53.0	16.6	8.0
Meat	89.0	80.4	71.5	79.7	80.7	64.0	90.3	76.7	69.0	61.0	21.5
Soy flour	83.8	70.6	59.2	91.4	77.0	70.2				57.0	22.5
Wheat germ	85.8	88.8	76.2	89.2	77.7	69.0				54.2	24.5
Bread	90.8	70.0	63.5							17.5	11.0

Man and the rat appeared to react in nearly identical fashion to the addition of meat to the diet. Digestibility was not improved, but the figures for biologic value were increased by approximately 10 per cent. The dogs, however, apparently digest the meat-supplemented diet better in addition to showing an increase in biologic value of 10 per cent. The overall net gain for the dogs was thus considerably more than in the other two species, 16 per cent compared to 8 per cent.

The data on man and the rats do not agree on the soy flour-supplemented diet. Whereas no improvement was noted by this addition for human beings in either biologic value or digestibility, both of these showed improvement in



the rats. Wheat germ supplements, while resulting in a gain in the net protein value of like amounts in the two species, showed the improvement in man entirely in biologic value whereas it was greatest in digestibility for the rat. As has been pointed out,<sup>1</sup> the data on soy flour and wheat germ in man are much less extensive than on the basal or meat-supplemented diet. Thus, we are unable to state that the apparent species differences in these two proteins are real.

The results on young growing rats are similar to those obtained with adult rats and, except for soy flour, also for those obtained with man. Supplements of meat, soy flour, or wheat germ apparently were of essentially equal effectiveness as they were in the adult animals. As in man, bread additions neither improved nor decreased the nutritive value. It is of interest that whereas meat supplementation caused an improvement of 8 per cent in the net protein value for adult animals, the relative improvement was much greater in the growing animals.

Recently Mitchell and Block<sup>7</sup> have presented convincing evidence that the biologic value of proteins as determined with rats bears a close relationship to their amino acid composition and may be estimated from the amino acid content. If, as suggested by the present data, the rat and man react similarly to various proteins, the value of the available data on proteins determined with rats is considerably enhanced. It further suggests that the percentage composition of the ideal protein for the two species is similar and lends support to the method of calculating amino acid requirements previously proposed by Stare, Hegsted, and McKibbin<sup>8</sup> and extended by Harte and Travers.<sup>9</sup> Since growing rats apparently classify proteins in a similar manner to adult rats, the results of such simple feeding experiments may be applicable for the evaluation of human dietaries as well.

#### CONCLUSION

Human subjects, rats, and dogs have been used to determine the biologic value of proteins in natural diets. The rat appeared to react in an exactly comparable manner to the human being when meat was added to the diet. Digestibility was not improved but the biologic value was increased 10 per cent. With the dog, on the other hand, both digestibility and biologic value were improved by meat supplementation.

The rat in these studies appeared more comparable to the human being than the dog.

Young growing rats, either by paired or ad libitum feeding, appear to classify these proteins in a similar manner to adult rats.

#### REFERENCES

1. Hegsted, D. M., Tsongas, A. G., Abbott, D. B., and Stare, F. J.: Protein Requirements of Adults, *J. LAB. & CLIN. MED.* 31: 261, 1946.
2. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B.: Choline in the Nutrition of Chicks, *J. Biol. Chem.* 138: 459, 1941.

3. Allison, J. B., and Anderson, J. S.: The Relation Between Absorbed Nitrogen, Nitrogen Balance, and Biological Value of Proteins in Adult Dogs, *J. Nutrition* 29: 413, 1945.
4. Hegsted, D. M., and Worcester, J.: A Study of the Relation Between Protein Efficiency and Gain in Weight on Diets of Constant Protein Intake, *J. Nutrition*. In press.
5. Hegsted, D. M.: The Minimum Protein Requirements of Adults. Symposium on Body Proteins, the University of Buffalo Centennial, 1946, University of Buffalo Press. In Press.
6. Bosshardt, D. K., and Barnes, R. H.: The Determination of Metabolic Fecal Nitrogen and Protein Digestibility, *J. Nutrition* 31: 13, 1946.
7. Mitchell, H. H., and Block, R. J.: Some Relationships Between the Amino Acid Contents of Proteins and Their Nutritive Values for the Rat, *J. Biol. Chem.* 163: 599, 1946.
8. Stare, F. J., Hegsted, D. M., and McKibbin, J. M.: Nutrition, *Ann. Rev. Biochem.* 14: 431, 1945.
9. Harte, R. A., and Travers, J. J.: Human Amino Acid Requirements, *Science* 105: 15, 1947.

# PROTHROMBIN TIME STUDIES ON HUMAN BEINGS AND EXPERIMENTAL ANIMALS

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THE one-stage assay of prothrombin has been shown to be affected by the concentration of fibrinogen.<sup>1</sup> By diluting a given plasma with a fibrinogen solution, sharper end points are realized at the higher dilutions, and the assay range may be extended to considerably higher plasma dilutions than is possible when the dilution of the substrate is not corrected. Preliminary *in vitro* prothrombin time studies had suggested that plasma fibrinogen concentrations might introduce changes in the apparent prothrombin levels as measured on saline diluted samples. The work reported here entails a study of the prothrombin time variable in experimental animals and in human beings as reflected by the clotting times of saline and fibrinogen dilutions of plasma in relation to the actual plasma fibrinogen level.

## EXPERIMENTAL

All prothrombin determinations were carried out according to the method outlined by Deutsch and Gerarde.<sup>1</sup> The bovine fibrinogen was prepared according to the method of Seegers and associates.<sup>2</sup> It was used in the form of a 0.3 per cent solution (clottable fibrinogen) at pH 7.2 to 7.4. The major portion of the thromboplastin used in this work was prepared from rabbit brain according to the method of Quick.<sup>3</sup> One preparation however, was made according to the method of Link and associates.<sup>4</sup> After extraction of the thromboplastin with saline according to Quick's<sup>3</sup> method, an equal volume of a pH 7.4 buffer that was 0.05 M in cacodylate and diethyl barbiturate and 0.025 M in  $\text{CaCl}_2$  was added. This mixture was centrifuged lightly and 1.0 c.c. portions of the supernatant were added to 0.5 c.c. of various saline or fibrinogen dilutions of a given plasma. The length of time for clotting at 25 degrees ( $\pm 1$  degree) was determined visually. All plasmas were obtained by mixing 9 c.c. of blood with 1 c.c. of 0.1 M sodium oxalate, the resultant oxalated plasma being considered 100 per cent according to the usual convention. Fibrinogen assays were carried out by the method as outlined by Reiner,<sup>5</sup> the fibrin nitrogen being estimated by micro-Kjeldahl or colorimetrically following nesslerization.

Rats used for observations on the effect of caffeine, sodium salicylate, and vitamin K on prothrombin time were maintained two weeks or longer prior to the experiment on the relatively low vitamin K diet as used by Link and co-workers.<sup>6</sup> The rabbits used were fed exclusively on a commercial rabbit-pellet diet. All drugs with the exception of vitamin K were administered by stomach tube; the latter compound was given by intraperitoneal injection.

## RESULTS

*Caffeine Effects.*—In the higher saline dilutions of plasma (10 per cent plasma) the small amount of fibrinogen present makes difficult the determination of the appearance of the relatively small fibrin clot formed. A plasma to which

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various levels of fibrinogen have been added prior to saline dilution forms clots at 10 per cent plasma concentrations that are more readily discerned although no additional prothrombin has been added to the system.<sup>1</sup> It also has been indicated previously that the ingestion of caffeine by rabbits may alter the prothrombin time of saline dilutions of such plasma by virtue of its raising the fibrinogen level. In further testing this hypothesis rabbits were fed caffeine citrate at the level of 100 mg. per kilogram, and the prothrombin times of saline and fibrinogen dilutions of the plasma as well as the plasma fibrinogen levels were determined before and after such medication. Little variation in the prothrombin times of saline- and fibrinogen-diluted plasma was seen at the higher plasma concentrations (80 to 10 per cent). Therefore, prothrombin times were determined only for a 10 per cent dilution of plasma in 0.15 M NaCl and for 10 and 1 per cent dilutions in 0.3 per cent fibrinogen. Saline dilutions of plasma in excess of 1:10 (10 per cent plasma) were not carried out due to the difficulty and often impossibility of determining the end point of the clotting reaction. The results of these determinations on eight rabbits are shown in Table I. In addition, eight male rats weighing from 250 to 300 grams, which had been maintained on the relatively low vitamin K diet of Link and co-workers<sup>6</sup> for at least two weeks, were given 100 mg. of caffeine citrate per kilogram. The findings for these rats are shown in the caffeine data of (Table II). The marked increases in plasma fibrinogen values of these rats were found forty-eight hours after the ingestion of caffeine citrate. It can be seen from the data that the plasma fibrinogen levels vary considerably after treatment; in general, a distinct rise was noted which is in agreement with the work of Field and associates.<sup>7</sup> Although the 10 per cent dilutions of rabbit plasma in saline tend toward slightly shorter prothrombin times due to the enhanced fibrinogen levels, the effect is by no means clear-cut. Rat plasma showed slightly lengthened values in all dilutions as compared with the controls (Table II). The marked increases in plasma fibrinogen values of these rats were not reflected in shortened prothrombin times of the 10 per cent plasma in saline as would be expected from the differences of the saline and fibrinogen

TABLE I. EFFECT OF CAFFEINE ON FIBRINOGEN LEVELS AND PROTHROMBIN TIME OF RABBITS

RABBIT	FIBRINOGEN CHANGE IN MILLI- GRAMS PER CENT*	PROTHROMBIN TIME (SECONDS)								
		10 PER CENT PLASMA IN 0.9 PER CENT SALINE			10 PER CENT PLASMA IN 0.3 PER CENT FIBRINOGEN			1 PER CENT PLASMA IN 0.3 PER CENT FIBRINOGEN		
		BEFORE	AFTER†	CHANGE	BEFORE	AFTER†	CHANGE	BEFORE	AFTER†	CHANGE
1	+168	23.8	21.9	-1.9	19.1	19.1	0	43.3	61.2	+17.9
2	+220	49.4	22.4	-27.0	18.9	17.1	-1.8	46.2	45.8	-0.4
3	+7	24.6	25.0	+0.4	20.4	21.1	+0.7	55.3	72.1	+16.8
4	+138	28.8	22.8	-6.0	20.6	21.0	+0.4	59.2	65.0	+5.8
5	+32	24.2	27.0	+2.8	20.2	20.9	+0.7	57.9	71.3	+13.4
6	-68	28.6	27.6	-1.0	20.1	21.0	+0.9	60.3	68.1	+7.8
7	+151	21.1	15.6	-4.6	16.5	15.6	-0.9	79.8	82.9	+3.1
8	+178	16.5	13.5	-3.0	13.4	13.4	0.0	45.7	60.7	+15.0

\*Change in fibrinogen levels forty-eight hours after administration of 100 mg. caffeine citrate per kilogram.

†Prothrombin times forty-eight hours after the administration of 100 mg. caffeine citrate per kilogram.

TABLE II. EFFECT OF CAFFEINE, SODIUM SALICYLATE, AND VITAMIN K\* ON FIBRINOGEN LEVELS AND PROTHROMBIN TIME OF RATS

GROUP	NUMBER IN GROUP	MILLIGRAMS PER CENT FIBRINOGEN (RANGE)	AVERAGE PROTHROMBIN TIME (SECONDS)		
			10 PER CENT PLASMA IN 0.9 PER CENT SALINE (RANGE)	10 PER CENT PLASMA IN 0.3 PER CENT FIBRINOGEN (RANGE)	1 PER CENT PLASMA IN 0.3 PER CENT FIBRINOGEN (RANGE)
Controls	20	215.0 (184 to 362)	34.5 (30.6 to 39.6)	23.9 (20.4 to 27.5)	80 (57 to 98)
	1†	0.0	>300	22.8	47
	1†	0.0	>120	20.4	69
Sodium salicylate	10	202.3 (90 to 328)	60.3 (42.7 to 90.2)	35.4 (23.2 to 51.8)	120 (65 to 195)
Sodium salicylate and vitamin K*	10	319.0 (115 to 520)	35.2 (31.3 to 39.9)	25.0 (22.3 to 28.2)	83 (55 to 107)
Vitamin K*	10	408.4 (298.0 to 497.0)	39.3 (37.7 to 42.0)	30.8 (27.9 to 33.4)	83 (57 to 104)
Caffeine	8	502.0 (420.0 to 640.0)	39.4 (32.8 to 51.8)	29.4 (25.5 to 41.3)	93 (66 to 186)

\*The vitamin K used was Synkayvite (tetrasodium 2-methyl-1, 4-naphthohydroquinone diphosphoric ester).

†From a control group which showed no fibrinogen by the assay method used.

diluted systems of the 10 per cent rat plasma concentrations. The prothrombin times of the 1 per cent plasma in 0.3 per cent fibrinogen while indicating a mild hypoprothrombinemia do not fall in line with the results on rabbits (Table I), where even in the presence of a mild hypoprothrombinemia there were in general slightly shortened prothrombin times in the 10 per cent plasma concentration in saline of rabbits showing enhanced fibrinogen values.

As will be discussed later, there are various factors which may be tending to give results which are difficult to evaluate in the "one-stage" prothrombin assay. The interpretation by Field and associates<sup>8</sup> that shortening of prothrombin times as shown by some rabbits is due to an enhanced level of prothrombin is not borne out by the clotting times of 1 per cent plasma in fibrinogen. The lengthened prothrombin times of both rat and rabbit plasma at this dilution are indicative of a decreased amount of prothrombin as a result of caffeine ingestion. The results of the clotting times of 10 per cent rabbit plasma in saline are quite analogous to the results of Quick<sup>9</sup> who could demonstrate no hyperprothrombinemia as the result of caffeine feeding. Interesting is the report of Scherf and Schlachman<sup>10</sup> who found a rapid lowering of the prothrombin time of the plasma of patients who had received aminophyllin and theophyllin.

*Salicylate and Vitamin K Effects.*—It appeared that the hypoprothrombinemia experienced in rats as a result of salicylate ingestion<sup>6</sup> might likewise be a reflection of the assay technique as influenced by a lowered plasma fibrinogen level. Experiments studying the effect of sodium salicylate and vitamin K were carried out under conditions similar to those employed by Link and co-workers,<sup>6</sup> except for the inclusion of fibrinogen dilution prothrombin assay procedures.

These experiments were conducted on male rats weighing 250 to 300 grams and maintained under the same conditions as those on the caffeine experiment. The animals were given 100 mg. sodium salicylate by stomach tube and prothrombin studies were carried out approximately twenty hours later. The effect of such a level of sodium salicylate accompanied by an intraperitoneal injection of 5 mg. equivalents of vitamin K (Synkayvite: tetrasodium 2-methyl-1, 4-naphthohydroquinone diphosphoric ester), as well as the effect of vitamin K alone, was determined on individual groups of animals. The analytic findings resulting from this medication as compared with control rats receiving no treatment are shown in Table II.

It is apparent from the data that sodium salicylate induces a hypoprothrombinemia and that the effect observed by Link and associates<sup>6</sup> is not one of a decrease of fibrinogen level as suggested by Deutsch and Gerarde.<sup>1</sup>

Vitamin K counteracts the effect of the sodium salicylate; this effect does not appear to be due to the enhanced plasma fibrinogen level elicited by vitamin K administration. The data of Table II, however, in no way suggest that vitamin K can induce hyperprothrombinemia as has been stated by Field and Link<sup>11</sup> and Richards and Shapiro.<sup>12</sup> Mikhlin<sup>13</sup> has indicated that vitamins K<sub>1</sub> and K<sub>2</sub> as well as related synthetic products are without effect on the blood of normal animals and man. However, both Mikhlin<sup>13, 14</sup> and Babuk<sup>15</sup> report that a vitamin K concentrate prepared from maize stigmas accelerated the normal coagulability of the blood in the dog, rabbit, rat, and man. They attributed this effect to a hyperprothrombinemia, although in view of our findings it is possible that this noted effect may be due to other factors.

*Afibrinogenemic Rats.*—Interestingly in the control rats studied there appeared two afibrinogenemic animals. While 10 per cent saline dilution and even undiluted plasma failed to clot in the usual prothrombin assay, dilution of the plasma of these animals with fibrinogen gave prothrombin times which indicated normal or enhanced values of plasma prothrombin. It would appear that the diet used by Link<sup>6</sup> tends to produce low plasma fibrinogen levels. Similar responding animals have been found on this diet by Link.<sup>16</sup> These two animals showed no tendency toward hemorrhage as has been reported for afibrinogenemic human beings by various authors.<sup>17-20</sup> In fact, the animals studied here in whose plasma no fibrinogen could be demonstrated were able to survive heart puncture.

*Thromboplastin.*—In part, the discrepancy between the results of Field and associates<sup>8</sup> and of Quick<sup>9</sup> might be due to differences in the activities of the thromboplastin preparations used. We have found the prothrombin time of rabbit plasma at 25 degrees with the Quick<sup>3</sup> thromboplastin preparation to be shorter than that recorded by Link<sup>4, 8</sup> for rabbit plasma at 37 degrees. Although Quick<sup>9</sup> has likewise commented on the lowered activity of the Link<sup>4</sup> thromboplastin, we are not aware of a specific demonstration of such a difference. To test this experimentally, pooled macerated rabbit brains were divided into two equal portions and thromboplastin was prepared by both the method of Link<sup>4</sup> and that of Quick.<sup>3</sup> The prothrombin times of fibrinogen and saline

dilutions of rabbit and human plasma were determined using these thromboplastin preparations under identical conditions. The results for human plasma which were similar to those found for rabbit plasma are shown in Fig. 1 and clearly indicate the greater activity of the Quick<sup>2</sup> thromboplastin preparation. In this respect Scherf and Schlachman<sup>19</sup> used Russell viper venom as the thromboplastin which in comparison with the Quick thromboplastin is relatively low in activity. From this it appears that low activity thromboplastins may possibly show up plasma fibrinogen increases as apparent prothrombin increases. Prothrombin measurements of such plasma should be carried out at high plasma dilution in the presence of a constant substrate (fibrinogen) level if the results of the one-stage prothrombin time assay method are to be interpreted in terms of prothrombin content.

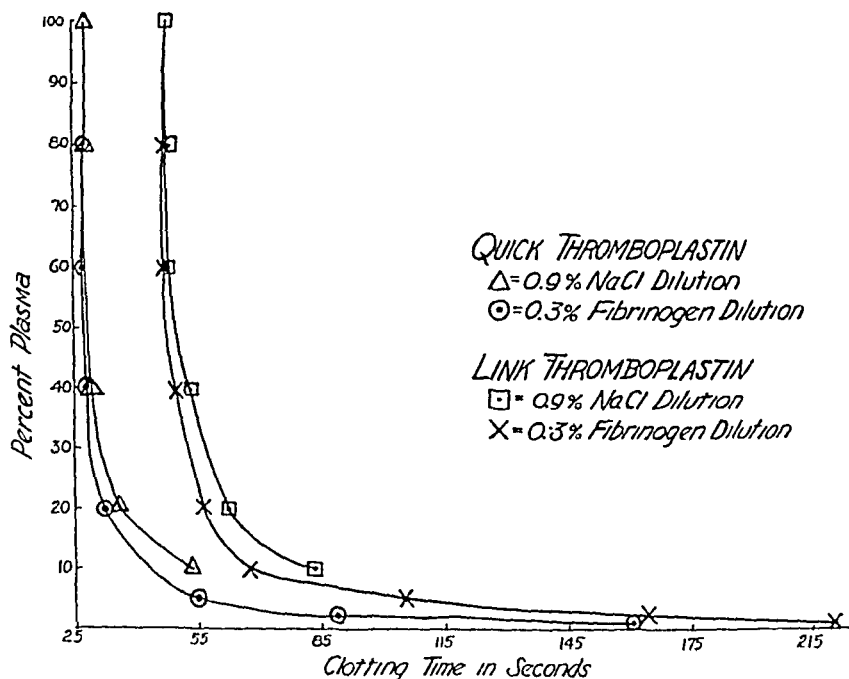


Fig. 1.—Comparison of clotting times of saline and fibrinogen dilutions of a single human plasma using Link<sup>4</sup> and Quick<sup>2</sup> thromboplastin.

*Prothrombin Variations in Human Beings.*—The relatively wide variations in prothrombin levels for essentially normal rats and rabbits as reflected by the clotting time of their 1 per cent solution of plasma in fibrinogen are interesting. It has been claimed by Quick<sup>21</sup> that the prothrombin levels of normal human beings fall within a narrow range. Other authors, however, indicate that considerable variations exists.<sup>22, 23</sup> Warner and associates<sup>24</sup> indicate that the effects of plasma antithrombin are minimized by high dilution of the plasma. Prothrombin assays at a plasma concentration of 1 per cent in the presence of a constant level of fibrinogen are probably more indicative of prothrombin content than assays

TABLE III. PROTHROMBIN TIME AND FIBRINOGEN LEVELS OF NORMAL HUMAN BEINGS ARRANGED ACCORDING TO INCREASING LEVELS OF FIBRINOGEN

PATIENT	MILLIGRAMS PER CENT FIBRINOGEN	PROTHROMBIN TIME (SECONDS)		
		10 PER CENT PLASMA IN 0.9 PER CENT SALINE	10 PER CENT PLASMA IN 0.3 PER CENT FIBRINOGEN	1 PER CENT PLASMA IN 0.3 PER CENT FIBRINOGEN
1	190	44.3	40.0	193
2	207	40.1	32.9	186
3	220	33.0	30.3	176
4	225	41.1	36.5	144
5	260	36.6	32.7	198
6	272	39.5	25.2	141
7	277	35.8	33.1	180
8	280	38.5	34.4	156
9	297	37.7	32.5	147
10	303	37.7	33.7	161
11	304	38.3	32.8	128
12	311	35.3	32.5	121
13	317	44.2	38.2	177
14	319	38.2	32.1	158
15	327	34.4	34.6	161
16	343	39.8	32.7	148
17	344	36.8	35.4	162
18	346	38.0	34.4	144
19	347	36.2	31.0	143
20	376	38.1	34.3	159
21	432	39.2	37.1	192
22	439	36.1	31.4	142
Average	306	38.1	33.5	160
Range	(190 to 439)	(34.4 to 44.3)	(25.2 to 40.0)	(124 to 198)

conducted in relatively concentrated plasma or in saline dilutions where the enzyme substrate tends to become the limiting factor. Application of the fibrinogen dilution technique was applied to the plasmas of a series of normal human subjects. Considerable differences in the prothrombin levels as reflected by variations in the prothrombin time were found by assay of 1 per cent plasmas. The variations encountered in the saline-diluted 10 per cent plasma were relatively smaller. These data are shown in Table III. There appears to be little correlation between plasma fibrinogen levels and the variations in the prothrombin times of plasma diluted to a level of 10 per cent with saline or fibrinogen. In general, however, the fibrinogen-diluted plasmas showed a slightly shortened prothrombin time.

To determine whether markedly enhanced plasma fibrinogen levels would affect the prothrombin time, a series of clinical cases tending in this direction were studied. They represented varied pathologic conditions with plasma fibrinogen values extending from normal to extremely high levels as shown in Table IV. The prothrombin times of 1 per cent plasma in fibrinogen were analogous to those found in normal individuals. However, the individual variations encountered were much greater. This was true also for the 10 per cent plasma clotting times. A noticeable difference in many cases was the lengthening of the prothrombin times of the 10 per cent plasmas diluted with fibrinogen as contrasted to the saline dilution. This was particularly evident in the samples of plasma having the higher fibrinogen levels. In the normal series,



TABLE IV. PROTHROMBIN TIMES AND FIBRINOGEN LEVELS OF HUMAN BEINGS WITH VARIOUS DISEASES ARRANGED ACCORDING TO INCREASING LEVELS OF FIBRINOGEN

PATIENT	DIAGNOSIS	MILLIGRAMS PER CENT FIBRINOGEN	PROTHROMBIN TIME (SECONDS)		
			10 PER CENT PLASMA IN 0.9 PER CENT SALINE	10 PER CENT PLASMA IN 0.3 PER CENT FIBRINOGEN	1 PER CENT PLASMA IN 0.3 PER CENT FIBRINOGEN
1	Atrophic cirrhosis	288	60.2	55.8	277.0
2	Carcinoma, uterus; x-ray therapy	288	34.4	30.5	129
3	Tuberculosis	298	47.5	47.3	239
4	Carcinoma, uterus; x-ray therapy	309	36.8	32.5	140
5	Atrophic cirrhosis	310	41.4	38.6	203
6	Chronic ileitis	313	44.0	41.4	253
7	Infectious hepatitis	320	40.7	39.6	248
8	Carcinoma, colon	336	46.3	45.5	203
9	Anxiety tension state	354	40.2	37.5	149
10	Sinusitis and otitis media	392	39.6	36.4	133
11	Osteomyelitis	393	38.1	31.2	122
12	Lues with retinitis	393	37.3	35.4	205
13	Carcinoma, breast	403	35.2	33.4	154
14	Tuberculosis	410	43.0	44.2	106
15	Carcinoma, uterus; x-ray therapy	413	33.9	29.8	131
16	Furunculosis	416	39.8	34.1	96
17	Atrophic cirrhosis	435	47.2	44.6	215
18	Myelogenous leucemia	444	46.1	47.2	201
19	Carcinoma, larynx	470	36.9	35.3	169
20	Rheumatic fever	477	33.7	31.8	137
21	Multiple myeloma	489	36.3	34.2	96
22	Carcinoma, bladder	490	36.7	32.6	148
23	Tuberculosis	525	41.1	45.6	226
24	Tuberculosis	540	35.6	34.2	155
25	Carcinoma, uterus; x-ray therapy	549	29.2	27.6	107
26	Carcinoma, breast; x-ray therapy	560	30.9	30.0	130
27	Arteriosclerotic gangrene	586	33.2	30.9	133
28	Subdiaphragmatic abscess	589	33.3	33.6	146
29	Hypertensive heart disease	591	42.2	42.5	244
30	Carcinoma, esophagus	594	36.4	35.5	201
31	Carcinoma, uterus; x-ray therapy	610	37.4	38.0	132
32	Peritonitis	615	43.0	43.4	153
33	Cholecystitis with lithiasis	619	41.0	43.5	193
34	Carcinoma, stomach	637	34.4	35.8	139
35	Constrictive pericarditis	657	38.5	41.7	202
36	Epididymitis	672	44.0	45.7	172
37	Carcinoma, prostate	691	31.9	29.7	125
38	Multiple burns	754	32.3	33.3	138
39	Xanthochromatosis	777	37.0	38.5	161
40	Thrombophlebitis	778	33.0	31.3	116
41	Carcinoma, esophagus	820	34.4	36.4	141
42	Bursitis and tendonitis	828	35.7	33.2	125
43	Rheumatic fever	1,040	31.7	33.4	142
Average		522.6	38.4	37.2	164
Range		(288 to 1,040)	(29.2 to 60.2)	(27.6 to 55.8)	(96 to 277)

as previously noted in all except one instance, the prothrombin times of 10 per cent plasma diluted with fibrinogen were shorter than the corresponding saline dilutions.

In previous in vitro studies fibrinogen was added to plasma to provide a system containing variable amounts of fibrinogen with a constant level of prothrombin.<sup>1</sup> Dilution of such a plasma with saline shows that the fibrinogen addition definitely affects the apparent prothrombin level as indicated by shorter prothrombin times in the higher dilutions. In our present work similar high fibrinogen level plasmas as occurring in various pathologic conditions were studied. The results (Table IV) are difficult to appraise. It would appear that fibrinogen fluctuations might be accompanied by variation in such clotting entities as inhibitors and prothrombin. A possible factor tending to introduce variations is the rate at which prothrombin is converted to thrombin. Thus, the discrepancies of prothrombin content of various animal plasmas as determined by the one-stage method of Quick<sup>21</sup> and the two-stage method of Warner and associates<sup>25</sup> appear to be conversion rate differences rather than absolute prothrombin levels. It is possible that even within a given species this conversion rate may vary. In addition, the presence of inhibitors for prothrombin, thrombin, and thromboplastin likewise affect clotting reactions. The true evaluation of the blood clotting mechanism as revealed by "one-stage" prothrombin assays should take into account all of these factors as well as the factors we have studied. Since simultaneous control of all recognized clotting factors in the "one-stage" type of assay would be extremely difficult, it would appear that assay techniques built around the "two-stage" assay would be more feasible for the true evaluation of prothrombin. Here individual reactions can be somewhat segregated, controlled, and studied independently. The "one-stage" clotting picture represents the summation of various reactions, and it is difficult to appraise the effect of any one given reaction.

#### SUMMARY

Various experimental and clinical conditions involving variation in fibrinogen and prothrombin levels have been studied.

The apparent hyperprothrombinemia experienced by some workers as a result of caffeine feeding appeared as a mild hypoprothrombinemia when prothrombin time assays were carried out at a 1 per cent plasma concentration in the presence of 0.3 per cent fibrinogen. The effect of sodium salicylate on rats is to induce a definite hypoprothrombinemia with little alteration in fibrinogen level. Vitamin K while counteracting the action of sodium salicylate *does not* in itself produce hyperprothrombinemia. A series of normal human subjects and clinical patients showed relatively wide variations in prothrombin levels as revealed by prothrombin times of 1 per cent dilutions of plasma. The marked fluctuation in plasma fibrinogen content did not correlate with expected prothrombin times of saline dilutions; this indicates a need for the evaluation of clotting factors in addition to those studied here.

## REFERENCES

1. Deutsch, H. F., and Gerarde, H. W.: Biophysical Studies of Blood Plasma Proteins. V. The Effect of Fibrinogen on Prothrombin Time, *J. Biol. Chem.* 166: 381, 1946.
2. Seegers, W. H., Neft, M. L., and Vandenbelt, J. M.: Decomposition Products of Fibrinogen and Fibrin, *Arch. Biochem.* 7: 15, 1945.
3. Quick, A. J.: The Thromboplastin Reagent for the Determinations of Prothrombin, *Science* 92: 113, 1940.
4. Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P.: Studies on the Hemorrhagic Sweet Clover Disease. II. The Bioassay of Hemorrhagic Concentrates by Following the Prothrombin Level in the Plasma of Rabbit Blood, *J. Biol. Chem.* 138: 463, 1941.
5. Reiner, M.: *Manual of Clinical Chemistry*, New York, 1941, Interscience Publishers, Inc.
6. Link, K. P., Overman, R. S., Sullivan, W. R., Huebner, W. R., and Scheel, L. D.: Studies on the Hemorrhagic Sweet Clover Disease. XI. Hypoprothrombinemia in the Rat Induced by Salicylic Acid, *J. Biol. Chem.* 147: 463, 1943.
7. Field, J. B., Sveinbjörnsson, A., and Link, K. P.: Increased Plasma Fibrinogen Induced by Methylxanthines, *J. Biol. Chem.* 159: 525, 1946.
8. Field, J. B., Larsen, E. G., Spero, L., and Link, K. P.: Studies on the Hemorrhagic Sweet Clover Disease. XIV. Hyperprothrombinemia Induced by Methylxanthines and Its Effect on the Action of 3,3'-Methylenebis-(4-Hydroxycoumarin), *J. Biol. Chem.* 156: 725, 1944.
9. Quick, A. J.: Experimentally Induced Changes in the Prothrombin Level of the Blood. I. Quantitative Studies in Dogs Given Dicumarol; II. The Effect of Methylxanthines on Prothrombin per se and When Administered With Dicumarol, *J. Biol. Chem.* 161: 33, 1945.
10. Scherf, D., and Schlachman, M.: The Effect of Methylxanthines on the Prothrombin Time and the Coagulation of the Blood, *Am. J. M. Sc.* 212: 83, 1946.
11. Field, J. B., and Link, K. P.: Notes on Hyperprothrombinemia Induced by Vitamin K, *J. Biol. Chem.* 156: 739, 1944.
12. Richards, R. K., and Shapiro, S.: Experimental and Clinical Studies on the Action of High Doses of Hykinone and Other Menadione Derivatives, *J. Pharmacol. & Exper. Therap.* 84: 93, 1945.
13. Mikhlin, D. M.: Characteristics of the Antihemorrhagic Factor From Maize Stigmata (Vitamin K<sub>3</sub>), *Biokhimiya* 8: 158, 1943; *Chem. Abstr.* 38: 1775, 1944.
14. Mikhlin, D. M.: *Compt. rend. Acad. d. sc. URSS* 39: 277, 1943; *Chem. Abstr.* 37: 6711, 1943.
15. Babuk, V. V.: *Compt. rend. Acad. d. sc. URSS* 39: 277, 1943; *Chem. Abstr.* 38: 4014, 1944.
16. Link, K. P.: Personal communication.
17. Rabe, R., and Salomon, E.: Ueber Faserstoffmangel im Blute bei einem Falle von Hämophilis, *Deutsches Arch. f. klin. Med.* 132: 240, 1920.
18. Opitz, H., and Frei, M.: Pseudohemophilia in Female Infants, *Jahrb. f. Kinderh.* 94: 374, 1921.
19. Schönholzer, G.: Die hereditäre Fibrinogenopenie, *Deutsches Arch. f. klin. Med.* 184: 496, 1939.
20. Glanzmann, E., Steiner, H., and Keller, H.: Konstitutionelle angeborene Afibrinogenämie und Fibrinopenie im Kindesalter, *Schweiz. med. Wehnschr.* 70: 1243, 1261, 1940.
21. Quick, A. J.: *The Hemorrhagic Diseases*, Springfield, Ill., 1942, Charles C Thomas, Publisher.
22. Hause, W. A., and Tocantins, L. M.: Determination of Plasma Prothrombin Variations in Normal Men and Women, *Am. J. Clin. Path.* 11: 54, 1941.
23. Aggeler, P. M., Howard, J., Lucia, S. P., Clark, W., and Astaff, A.: Standardization of the Quick Prothrombin Test, *Blood* 1: 220, 1946.
24. Warner, E. D., Brinkhous, K. M., and Smith, H. P.: A Quantitative Study on Blood Clotting; Prothrombin Fluctuations Under Experimental Conditions, *Am. J. Physiol.* 114: 667, 1936.
25. Warner, E. D., Brinkhous, K. M., and Smith, H. P.: Plasma Prothrombin Levels in Various Vertebrates, *Am. J. Physiol.* 125: 296, 1939.

# THE CORPUSCULAR CONSTANTS OF COLLEGE WOMEN OF THE NORTH CENTRAL STATES\*

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AND EVA G. DONILSON||

**H**EMATOLOGIC data on 4,550 normal college women, 16 to 30 years old, inclusive, were reported recently by Ohlson and associates.<sup>1</sup> Included in these were data from 1,348 individuals, 17 to 24 years old, inclusive, on whom the hemoglobin concentration, the number of red cells, and the packed cell volume were determined on the same samples of blood. From these values, which include data from the nutrition laboratories of Iowa State College, Kansas State College, University of Minnesota, and Oklahoma Agricultural and Mechanical College, the corpuscular constants of Wintrobe<sup>2</sup> have been calculated. Complete information on the experimental methods used in the study were given in the previously mentioned report.<sup>1</sup>

The corpuscular constants (mean corpuscular hemoglobin, mean corpuscular volume, and mean corpuscular hemoglobin concentration) were first introduced and defined by Wintrobe<sup>2</sup> in 1929. In 1934 he<sup>2</sup> summarized the data available at that time and reported values for the corpuscular constants on approximately 700 individuals, 274 of whom were normal women between the ages of 18 and 30 years, inclusive, from different parts of the United States and Denmark. The mean values for the women of this age range were: mean corpuscular hemoglobin, 28.8 micromicrograms; mean corpuscular volume, 87.0 cubic microns; and mean corpuscular hemoglobin concentration, 33.1 per cent. Since 1934 the corpuscular constants have been determined for subjects of both sexes in different parts of the world.<sup>4-14</sup> In 1942, while referring to values for corpuscular constants found since 1934, Wintrobe<sup>15</sup> stated, "Reports made since that time, with one exception, are either in exact agreement with those given or differ so slightly that the mean values remain the same when these additional data are used."

The present study of the corpuscular constants of 1,348 college women offers the largest series of values reported to date.

## RESULTS AND DISCUSSION

In Table I are given the number of subjects and the mean values for mean corpuscular hemoglobin, mean corpuscular volume, and mean corpuscular hemoglobin concentration for each group of women studied and for the subjects as a

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TABLE I. MEAN VALUES FOR MEAN CORPUSCULAR HEMOGLOBIN, MEAN CORPUSCULAR VOLUME, MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION, STANDARD DEVIATIONS OF MEANS (S), AND STANDARD ERRORS OF MEANS ( $S_x$ ) OF COLLEGE WOMEN OF FOUR STATES, WITH APPENDED VALUES FOR THESE CORPUSCULAR CONSTANTS AS REPORTED BY WINTROBE FOR WOMEN OF SIMILAR AGE RANGE

STATE	SUB- JECTS	M.C.H.			M.C.V.			M.C.H.C.		
		MEAN ( $\gamma\gamma$ )	S	$S_x$	MEAN $\mu^3$	S	$S_x$	MEAN (%)	S	$S_x$
Iowa	593	28.8	3.0	0.12	89.5	4.2	0.17	32.2	2.4	0.10
Kansas	220	29.1	2.3	0.16	91.7	4.8	0.32	31.7	2.2	0.15
Minnesota	292	30.7	2.1	0.12	92.7	6.6	0.39	33.2	1.8	0.11
Oklahoma	243	29.2	2.4	0.15	88.3	5.2	0.33	33.1	2.3	0.15
All four states	1348	29.3			90.3			32.5		
Wintrobe's report on women from the United States and Denmark	274	28.8			87.0			33.1		

whole. The standard deviations and the standard errors of the means are also included for each state group of subjects. For ease of comparison the values for corpuscular constants of the women between the ages 18 and 30 years, inclusive, given in Wintrobe's 1934 report have been included in the table.

The mean values of the corpuscular constants are somewhat different for the four groups of women studied. The respective maximum and minimum mean values are: mean corpuscular hemoglobin, 30.7 and 28.8 micromicrograms in Minnesota and Iowa, respectively; mean corpuscular volume, 92.7 and 88.3 cubic microns in Minnesota and Oklahoma, respectively; and mean corpuscular hemoglobin concentration, 33.2 and 31.7 per cent in Minnesota and Kansas, respectively. It is interesting to observe that the Minnesota group, in which the bloods were taken uniformly under basal conditions, show the highest values for all three corpuscular constants, although the mean values for hemoglobin, number of red cells, and packed red cell volume were the lowest of any state group.<sup>1</sup> Only in one of the four states, Iowa, is the mean corpuscular hemoglobin as low as 28.8 micromicrograms, the mean value reported for women of a similar age range by Wintrobe. In no instance is the mean corpuscular volume as low as Wintrobe's 87.0 cubic microns. In two states, Minnesota and Oklahoma, the values for mean corpuscular hemoglobin concentration are practically the same as the 33.1 per cent reported by Wintrobe, while in the other two states lower values are found.

A study of the significance of the differences between the mean values for the four groups shows that significant differences occur for all three corpuscular constants (Table II). For mean corpuscular hemoglobin, three out of six possible comparisons show highly significant differences between means, that is, critical ratios of 3.0 or more. Highly significant differences are found in five out of six instances for mean corpuscular volume and in four out of six for mean corpuscular hemoglobin concentration.

Even though there are significant differences between the means for each constant, there seems to be ample justification for putting the data of the four state groups together to obtain mean values of the constants for the 1,348 subjects as a whole. The reasons that prompt such a conclusion are (1) the marked inter- and intraindividual variations observed in the different states during the

TABLE II. SIGNIFICANCE OF DIFFERENCE BETWEEN MEANS FOUND IN RESPECTIVE STATE GROUPS FOR MEAN CORPUSCULAR HEMOGLOBIN, MEAN CORPUSCULAR VOLUME, AND MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION EXPRESSED AS CRITICAL RATIO\*

		OKLAHOMA	IOWA	KANSAS
Mean corpuscular hemoglobin	Minnesota	7.8	10.6	8.0
	Oklahoma		2.4	0.5
	Iowa			1.5
Mean corpuscular volume	Minnesota	8.0	7.4	2.0
	Oklahoma		3.2	7.4
	Iowa			6.1
Mean corpuscular hemoglobin concentration	Minnesota	0.5	6.7	8.0
	Oklahoma		5.0	6.6
	Iowa			2.8

$$*C.R. = \frac{m_1 - m_2}{S_x \text{ diff.}}, \text{ where } S_x \text{ diff.} = \sqrt{\frac{S_1^2}{N_1} + \frac{S_2^2}{N_2}}; m_1 \text{ and } m_2, S_1 \text{ and } S_2, \text{ and } N_1 \text{ and } N_2$$

denote the means, the standard deviations of the means, and the number of cases, respectively; and  $S_x \text{ diff.}$  stands for the standard error of the difference between the means.

present study and (2) the wide variation in the data used by Wintrobe to establish his means for these constants. The range of mean values used by Wintrobe was: 28 to 31 micromicrograms for mean corpuscular hemoglobin, 82 to 93 cubic microns for mean corpuscular volume, and 32 to 34 per cent for mean corpuscular hemoglobin concentration.

By reference to Table I, it is seen that the corpuscular constants for the subjects of the present study as a whole may be compared readily with the comparable values reported by Wintrobe for women of a similar age range. The respective values are: mean corpuscular hemoglobin, 29.3 and 28.8 micromicrograms; mean corpuscular volume, 90.3 and 87.0 cubic microns; and mean corpuscular hemoglobin concentration, 32.5 and 33.1 per cent. The similarity of these values in each case is quite striking, particularly so for mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration. When one considers that Wintrobe's range for mean corpuscular volume, 82 to 93 cubic microns, was much wider than for the other constants, the difference of 3.0 cubic microns would seem to have little, if any, greater significance than the differences found for the other constants.

This study and a previous one,<sup>1</sup> as well as numerous other reports in the literature, serve to emphasize the fact that the mean values for hemoglobin, red cell count, and packed red cell volume, and the mean corpuscular constants derived therefrom, show considerable variation between the different groups studied in this country and abroad. The lack of uniformity in these values, even when the numbers of subjects in the studies are large, leads to speculation as to the reasons for these differences. To what extent they are due to the experimental procedure employed and what role environmental conditions (temperature, humidity, altitude), race, and dietary factors play in their origin are points that are not clearly understood and require further elucidation.

#### SUMMARY

1. Values for mean corpuscular hemoglobin, mean corpuscular volume, and mean corpuscular hemoglobin concentration are reported on 1,348 women from

colleges or universities in four states, Iowa, Kansas, Minnesota, and Oklahoma.

2. The mean values found for these corpuscular constants are somewhat different for the four state groups. The respective maximum and minimum values are: mean corpuscular hemoglobin, 30.7 and 28.8 micromicrograms; mean corpuscular volume, 92.7 and 88.3 cubic microns; and mean corpuscular hemoglobin concentration, 33.2 and 31.7 per cent.

3. When the results on the 1,348 subjects are considered as a whole, the mean values for the corpuscular constants are 29.3 micromicrograms for mean corpuscular hemoglobin, 90.3 cubic microns for mean corpuscular volume, and 32.5 per cent for mean corpuscular hemoglobin concentration.

#### REFERENCES

1. Ohlson, M. A., Cederquist, D., Donelson, E. G., Leverton, R. M., Lewis, G. K., Himwich, W. A., and Reynolds, M. S.: Hemoglobin Concentrations, Red Cell Counts and Erythrocyte Volumes of College Women of the North Central States, *Am. J. Physiol.* 142: 727, 1944.
2. Wintrobe, M. M.: The Volume and Hemoglobin Content of the Red Blood Corpuscle, *Am. J. M. Sc.* 177: 513, 1929.
3. Wintrobe, M. M.: Anemia: Classification and Treatment on the Basis of Differences in the Average Volume and Hemoglobin Content of the Red Corpuscles, *Arch. Int. Med.* 54: 256, 1934.
4. Walters, O. S.: Normal Erythrocyte, Hemoglobin and Packed Cell Volume Standards in Young Men, *J. LAB. & CLIN. MED.* 19: 851, 1934.
5. Linneberg, L. L., and Schartum-Hansen, H.: Blodets hemoglobinnmengde og de røde blodlegemers antall og volum hos friske menn og kvinner, *Norsk mag. f. laegevidensk.* 96: 832, 1935.
6. Price-Jones, C., Vaughan, J. M., and Goddard, H. M.: Hematological Standards of Healthy Persons, *J. Path. & Bact.* 40: 503, 1935.
7. Belk, W. P., Curtis, E., and Wilson, M. K.: Erythrocyte Counts, Hemoglobin and Erythrocyte Volume in Normal Young Men and Women Residing in the Eastern United States, *Am. J. Clin. Path.* 6: 487, 1936.
8. Mugrage, E. R., and Andresen, M. I.: Values for Red Blood Cells of Average Infants and Children, *Am. J. Dis. Child.* 51: 775, 1936.
9. Andresen, M. I., and Mugrage, E. R.: Red Blood Cell Values for Normal Men and Women, *Arch. Int. Med.* 58: 136, 1936.
10. Navarro, R. J.: Hematology in Filipinos. II. Normal Mean Corpuscular Volume, Mean Corpuscular Hemoglobin and Mean Corpuscular Hemoglobin Concentration; the Various Normal Blood Indexes, *J. Philippine Islands M. A.* 17: 611, 1937.
11. Faergeman, P.: The Normal Values for Hemoglobin, Erythrocytes and Cell Volume Per Cent, and the Corpuscular Constants Derived Thereof, in Children Aged 8 to 14 Years. Examinations of 300 School Children of Stockholm, *Acta med. Scandinav.* 95: 566, 1938.
12. Hamre, C. J., and Wong, K. K. L.: Hematologic Values for Normal Children 3, 4 and 5 Years of Age Living in Hawaii, *Am. J. Dis. Child.* 60: 22, 1940.
13. Hamre, C. J., and Au, M. H.: Hematologic Values for Normal Healthy Men 16 to 25 Years of Age, *J. LAB. & CLIN. MED.* 27: 1231, 1942.
14. Lewis, R. C., Kinsman, G. M., Iliff, A., and Duval, A. M.: The Effect of Change of Altitude on the Corpuscular Constants of Wintrobe, *Am. J. Clin. Path.* 13: 208, 1943.
15. Wintrobe, M. M.: *Clinical Hematology*, Philadelphia, 1942, Lea & Febiger, p. 78.

## TWO POPULAR FALLACIES REGARDING Rh

### PRELIMINARY REPORT OF SOME THOUGHT-PROVOKING OBSERVATIONS

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WITH the tremendous mass of literature on the Rh factor which has accumulated in the seven years since its discovery, it is inevitable that certain fallacious ideas should have reached print and should persist in the minds of many of their readers. One or two of these fallacies have been repeated in one paper after another without an adequate investigation of their origin or of their accuracy.

It is the purpose of this paper to present a preliminary report of some observations made in this laboratory which would seem to indicate the fallaciousness of two commonly held beliefs regarding Rh.

*Fallacy 1: Only a Small Percentage of Rh-Negative Persons Can Become Sensitized by Transfusion With Rh-Positive Blood.*—Wiener<sup>1-3</sup> has stated variously that between one in fifty and one in twenty-five Rh-negative persons who are "exposed to the Rh antigen" become sensitized, and his statements have been repeated widely elsewhere. He further states<sup>4</sup> that the incidence of sensitization by Rh-positive pregnancy, and by transfusion, is about the same. Unger<sup>5</sup> goes so far as to say that "The fact is now recognized that when repeated transfusions of Rh-positive blood are given to an Rh-negative patient, antibody response to the Rh antigen develops in approximately one in twenty-five patients." Yet, neither Wiener nor Unger refers to experimental or clinical data as a basis for his statements.

Actually there is much recent evidence from several sources that sensitization may occur in many more than one in twenty-five Rh-negative individuals who receive Rh-positive blood. Diamond and co-workers,<sup>6</sup> in a series of 2,500 servicemen who had been transfused under wartime conditions without regard for Rh type, were able to demonstrate anti-Rh antibodies in the serum of about 50 per cent of the Rh-negative individuals. Ross,<sup>7</sup> in a smaller series, likewise found Rh antibodies in nearly 50 per cent of the Rh-negative bloods.

In a similar survey now under way in this laboratory, we have examined bloods of a group of servicemen who have at some time in the past received transfusions, presumably without the benefit of Rh typing. These bloods were classified as Rh-positive or Rh-negative, according to their reactions with standard Rh<sub>0</sub> typing serum. All the Rh negatives and the majority of the Rh positives were subtyped with sera of Rh' and Rh'' specificity. All sera were examined then for Rh antibodies according to the method of Diamond and Denton,<sup>8</sup> a single drop of serum being incubated in a small tube with a drop of

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2 per cent suspension of Rh<sub>1</sub>Rh<sub>2</sub> cells in 30 per cent bovine albumen. Parallel tests were set up using Rh-negative cells. Sera agglutinating the Rh-positive cells were then titrated, using the same cell suspension both in saline and in albumen. The serum dilutions for the latter were made in normal serum, as suggested by Levine and Bernstein.<sup>9</sup> For comparison, all sera showing anti-Rh activity were also tested with the blocking technique of Wiener.

The results closely approximate those of Diamond and of Ross. Of the first 122 bloods examined, twenty were negative for the Rh<sub>0</sub> factor; of these, two were type Rh' and one was Rh''. Of these twenty sera, eleven (or 55 per cent) contained Rh antibodies, all of the blocking or incomplete variety, and all were inactive against saline-suspended cells. Titers against Rh<sub>1</sub>Rh<sub>2</sub> cells in albumen ranged from 1:1 to 1:5120, while blocking titers ranged from 0 to 1:32. The findings are summarized in Table I.

TABLE I. TITERS WITH RH<sub>1</sub>RH<sub>2</sub> CELLS

SERUM	SALINE	ALBUMEN	BLOCKING
1	—	1:4	1:1
2	—	1:64	1:2
3	—	1:2	1:1
4	—	1:8	1:4
5	—	1:32	1:2
6	—	1:128	1:16
7	—	1:8	1:4
8	—	1:4	1:1
9	—	1:5120	1:32
10	—	1:64	1:4
11	—	1:1	—

It is evident that if these sera had been examined with saline-suspended cells only, all would have been discarded as containing no antibodies. Yet, many of these patients had had one or more transfusion reactions, a few quite severe. There can be little question that they were sensitized to the Rh factor by transfusion.

In evaluating these findings it should be kept in mind that in choosing the subjects for this survey, all patients were included who gave any history of receiving whole blood overseas, or at small hospitals in this country, regardless of the number of transfusions or the elapsed time since the last one. Most of them had not received blood for many months. It seems quite likely that if they had been examined sooner after their last transfusions, the percentage showing antibodies would have been even higher.

Further evidence of this sort comes from the work of Diamond<sup>6</sup> and of Wiener and Sonn-Gordon<sup>10</sup> in sensitizing Rh-negative volunteers by repeated injections of Rh-positive blood. Although there was a great deal of variation in the ease with which the volunteers were sensitized, the percentage who eventually developed antibodies when sensitizing doses were continued was extremely high.

It would appear then that we must discard the notion that only one in twenty-five Rh-negative persons who are transfused with Rh-positive blood may become sensitized. Rather, it must be assumed that every Rh-negative patient is capable of developing Rh antibodies and should be transfused only with Rh-

negative blood. Transfusion reactions due to Rh sensitization are probably much more common than often is realized. Most of them can and should be avoided.

*Fallacy 2: Blocking Antibodies Are Always of Rh<sub>0</sub> Specificity.*—In 1944 and 1945 Race,<sup>10</sup> Wiener,<sup>11</sup> and Diamond<sup>12</sup> independently reported the discovery of an antibody which, while it did not of itself agglutinate Rh-positive cells, specifically combined with them so as to prevent their agglutination by a known Rh agglutinating serum. This blocking antibody was described as specific for the Rh<sub>0</sub> (D) element of the cells, blocked Rh<sub>1</sub> cells behaving like Rh' cells, and blocked Rh<sub>2</sub> cells like Rh''. This specificity has repeatedly been emphasized in the literature. Wiener<sup>4</sup> has even suggested that this phenomenon be used in identifying antisera of unknown specificity, blocked Rh<sub>1</sub> and Rh<sub>2</sub> cells being used in place of Rh' and Rh'' which are often not available.

Several new techniques since have been developed to demonstrate these blocking antibodies by their active agglutination of Rh-positive cells in various viscous media, and these more sensitive methods have largely replaced the blocking test in routine use. Yet, there have been very few reports of further study of Rh antisera with these new techniques to determine whether blocking antibodies of other than pure Rh<sub>0</sub> specificity ever occur. Diamond and Abelson,<sup>13</sup> in describing their slide test for the blocking antibody, remarked that "Sera classified as anti Rh<sub>0</sub> are capable (by this technique) of agglutinating Rh' cells." Callender, and associates<sup>14</sup> also have described a serum with a blocking antibody of anti-c (Hr') specificity. In neither case, however, did the authors pursue the matter further.

It is then of interest to report the findings in an analysis of the anti-Rh sera encountered in our survey of transfused patients. Sera found to agglutinate Rh<sub>1</sub>Rh<sub>2</sub> cells in albumen were tested for specificity by the same technique, using Rh<sub>0</sub>, Rh', and Rh'' cell suspensions in albumen. The findings on the first ten sera are summarized in Table II.

TABLE II. SERA OF TRANSFUSED MEN; TITERS WITH VARIOUS TYPES OF CELLS IN SALINE AND 30 PER CENT BOVINE ALBUMEN

SERUM	CELL TYPE					
	Rh <sub>0</sub>		Rh'		Rh''	
	SALINE	ALBUMEN	SALINE	ALBUMEN	SALINE	ALBUMEN
1	—	1:4	—	1:1	—	—
2	—	1:64	—	—	—	—
3	—	1:2	—	—	—	—
4	—	1:8	—	1:1	—	—
5	—	1:32	—	1:1	—	—
6	—	1:64	—	1:4	—	1:1
7	—	1:8	—	—	—	—
8	—	1:4	—	—	—	—
9	—	1:5120	—	1:1280	—	—
10	—	1:32	—	1:1	—	1:1

It may be seen that, in spite of their complete lack of activity in saline, six of the ten sera had unmistakable activity against Rh' cells in albumen, and two against Rh''. These reactions were checked with the rabbit antihuman globulin technique of Coombs and associates,<sup>15</sup> with excellent agreement.

Furthermore, it was possible to demonstrate that Serum 9, with the highest titer of anti-Rh' antibodies, was capable of blocking Rh' cells in saline so that subsequently they could not be agglutinated by a standard anti-Rh' serum.

In order to determine whether these findings would be limited to patients sensitized by transfusions, ten unselected sera from Rh-negative women who had been sensitized by Rh-positive pregnancies were evaluated similarly. The findings are summarized in Table III.

TABLE III. SERA OF SENSITIZED RH-NEGATIVE WOMEN; REACTIONS WITH VARIOUS TYPES OF CELLS IN SALINE AND 30 PER CENT BOVINE ALBUMEN

SERUM	CELL TYPES					
	Rh <sub>0</sub>		Rh'		Rh''	
	SALINE	ALBUMEN	SALINE	ALBUMEN	SALINE	ALBUMEN
11	—	++	—	—	—	—
12	—	++++	—	++++	—	—
13	—	++++	—	—	—	—
14	++++	++++	—	++++	—	—
15	++++	++++	+	++++	—	—
16	—	++++	—	++++	—	—
17	+	++++	—	++++	—	—
18	—	++++	—	++++	—	+++
19	++++	++++	—	++	—	—
20	++++	++++	+	++++	—	—

It may be seen that whereas the usual Rh'-typing serum consists of a mixture of Rh' agglutinins and Rh<sub>0</sub>-blocking antibodies, quite the opposite situation is illustrated by Serum 14, a standard Rh<sub>0</sub> agglutinating serum which proved to contain Rh'-blocking antibodies as well. Considering the twenty sera examined, of the eighteen which had no activity against Rh' cells in saline, eleven (or 60 per cent) agglutinated these same cells in albumen, while two others with weak Rh' agglutinins clumped these cells very much more strongly in albumen. While none of the twenty showed Rh'' agglutinins in saline, three (or 15 per cent) agglutinated Rh'' cells in albumen.

It is realized that this series is small, but these preliminary observations appear sufficient to cast considerable doubt on the oft repeated statement that blocking antibodies are always of Rh<sub>0</sub> specificity.

#### SUMMARY

Two popular opinions regarding the Rh factors are discussed with evidence which suggests that they are fallacious:

1. *Only a small percentage of Rh-negative persons can become sensitized by transfusion with Rh-positive blood.* Evidence is presented that more than 50 per cent of such persons may develop antibodies. It is concluded that Rh-negative individuals should be transfused only with Rh-negative blood.

2. *Blocking antibodies are always of Rh<sub>0</sub> specificity.* Twenty antisera from Rh-negative persons sensitized by transfusion or by pregnancy were examined for activity against Rh' and Rh'' cells in albumen and in saline. Although none caused agglutination in saline, Rh' antibodies active in albumen were demonstrated in 60 per cent and Rh'' antibodies in 15 per cent.

## REFERENCES

1. Wiener, A. S.: The Rh Blood Types and Some of Their Applications, *Am. J. Clin. Path.* 15: 106, 1945.
2. Wiener, A. S.: Notes on the Pathogenesis of Congenital Hemolytic Disease (Erythroblastosis Fetalis), *Am. J. Clin. Path.* 16: 319, 1946.
3. Wiener, A. S.: Rh Factors in Clinical Medicine, *J. LAB. & CLIN. MED.* 30: 957, 1945.
4. Wiener, A. S.: Recent Developments in the Knowledge of the Rh-Hr Blood Types; Tests for Rh Sensitization, *Am. J. Clin. Path.* 16: 477, 1946.
5. Unger, L. J.: Sensitization of Rh Positive Patients by the Rh Factor, *J. LAB. & CLIN. MED.* 31: 793, 1946.
6. Diamond, L. K.: Physicochemical and Immunological Characteristics of Rh Antibodies, Read at the International Hematology and Rh Conference, Dallas, Tex., Nov. 16, 1946.
7. Ross, J. B.: Personal communication.
8. Diamond, L. K., and Denton, R. L.: Rh Agglutination in Various Media With Particular Reference to the Value of Albumen, *J. LAB. & CLIN. MED.* 30: 821, 1945.
9. Levine, P., and Bernstein, L.: Unpublished work referred to by Levine: The Present Status of the Rh Factor, *Am. J. Clin. Path.* 16: 597, 1946.
10. Race, R. R.: Incomplete Antibody in Human Serum, *Nature*, London 153: 771, 1944.
11. Wiener, A. S.: New Test (Blocking Test) for Rh Sensitization, *Proc. Soc. Exper. Biol. & Med.* 56: 173, 1944.
12. Diamond, L. K.: Importance of Rh Inhibitor Substance in Anti-Rh Serums, *J. Clin. Investigation* 24: 122, 1945.
13. Diamond, L. K., and Abelson, N. M.: The Demonstration of Anti-Rh Agglutinins—an Accurate and Rapid Slide Test, *J. LAB. & CLIN. MED.* 30: 204, 1945.
14. Callender, S. T., Race, R. R., and Paykoc, Z. V.: Sensitivity to Transfused Blood, *Brit. M. J.* 2: 83, 1945.
15. Coombs, R. R. A., Mourant, A. E., and Race, R. R.: A New Test for the Detection of Weak and "Incomplete" Rh Agglutinins, *Brit. J. Exper. Path.* 24: 255, 1945.
16. Wiener, A. S., and Sonn-Gordon, E. B.: Simple Method of Preparing Anti-Rh Serum in Normal Male Donors, *Am. J. Clin. Path.* 17: 67, 1947.

## PROGRESS

### METABOLIC COMPLEXITIES OF PELLAGRA

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THE current concepts of metabolism in pellagra commenced with the identification<sup>1</sup> of niacin as the blacktongue preventive principle of liver whose existence had been established by Goldberger and Wheeler<sup>2</sup> and the occurrence of this compound as part of codehydrogenases I and II.<sup>3, 4</sup> The numerous investigations reported since these papers have added many pieces of information, but the over-all picture is one which has grown steadily more perplexing. Although the fundamental significance of the pyridine nucleotides in cellular respiratory mechanisms is beyond dispute, attempts to correlate the behavior of such systems with the usual manifestations of niacin deficiency have met with no success.<sup>5, 6</sup> However, it does seem likely that failure of these systems is involved in the development of anemia in niacin-deficient dogs.<sup>7</sup>

The nature of the precipitating factor which results in blacktongue crises, characterized morphologically by lesions in the mucosa of the gastrointestinal tract and chemically by acidosis and dehydration, is as yet unknown. This crisis usually occurs within forty to sixty days after an adult dog is placed on a diet containing corn meal and the animal dies within a few days. Yet, the entire symptom complex disappears after a week of vigorous parenteral saline therapy, frequently never to return, although the animal survives for another three to six months.<sup>6</sup> While the niacin and coenzyme concentrations of the tissues of dogs in blacktongue are not reduced materially below normal, those of dogs successfully treated with salt solution, which survive several months longer, are dramatically reduced and death at this point does seem to be related to coenzyme deficiency. This situation scarcely seems compatible with simple niacin deficiency as the sole etiologic factor in the pathogenesis of blacktongue. It has been noted that the niacin content of the diets on which pellagra and blacktongue develop are not really remarkably low in niacin but contain an amount equal to that which must be added to prevent disease. Considering the rapid development of these diseases, this is a most unusual behavior for a simple vitamin deficiency. When it was found that the buccal manifestations of blacktongue did not appear for many months in dogs on a synthetic ration very low in niacin content, that when they did appear they were not accompanied by acidosis or dehydration, and that one-third of the dogs simply declined steadily and died after three to seven months in much the same manner as dogs on corn meal rations who have been successfully treated with salt solution, it was suggested by the present author<sup>8</sup>

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that corn is of positive etiologic significance in the development of blacktongue (and, presumably, of pellagra) and not merely a food of relatively low niacin content. While this was a revival of a time-honored, but hitherto unproved, thesis, it was based on experimental evidence. The same suggestion had been made earlier by Aykroyd and Swaminathan<sup>9</sup> after an appraisal of the niacin intake of pellagrous and nonpellagrous populations.

A new approach to this problem was offered by the finding that a suitable admixture of corn grits and a synthetic ration resulted in a suppression of rat growth which could be overcome by niacin<sup>10</sup> or by tryptophane.<sup>11</sup> At this writing, data are available concerning the effects of tryptophane on apparent niacin deficiency only in the rat and chick. This is particularly unfortunate since these findings in the rat make it difficult to understand the development of fatal niacin deficiency in the adult dog on purified rations, providing what would seem to be an otherwise adequate tryptophane intake (22 per cent casein)<sup>5</sup> and the use of almost identical rations as the basis for a niacin assay with pups.<sup>12</sup> Since tryptophane administration results in an increased urinary excretion of niacin derivatives, particularly N<sup>1</sup>-methylnicotinamide, in rats,<sup>13</sup> the possibility exists that tryptophane may serve as a precursor for niacin synthesis.

The presence of considerable quantities of a substance which has not been identified positively but with properties suggestive of quinolinic acid (pyridine, 2,3-dicarboxylic acid) in rat urine after tryptophane administration<sup>20</sup> offers a clue to the possible pathway of such a biologic synthesis of nicotinic acid from tryptophane. The observation that pyridoxine-deficient rats do not excrete extra N<sup>1</sup>-methylnicotinamide when given tryptophane<sup>14</sup> but do excrete abnormal quantities of xanthurenic acid,<sup>15</sup> a derivative of tryptophane, also makes a metabolic relationship between niacin and tryptophane seem likely. This defect in the metabolism of tryptophane in pyridoxine-deficient rats may be related to the demonstrated functioning of pyridoxine derivatives in decarboxylase<sup>16</sup> and transaminase systems.<sup>17</sup> This niacin-tryptophane-pyridoxine relationship seems all the more significant when one notes that there exists reason to suspect that the fluorescent compound, F<sub>1</sub>, whose excretion increases with the development of pellagra<sup>18</sup> may be the pyridoxic acid found in the urine of normal individuals after the administration of pyridoxine.<sup>19</sup>

It is somewhat disconcerting to note that increasing the casein intake of rats does not increase the N<sup>1</sup>-methylnicotinamide excretion in their urine.<sup>14</sup> However, this situation yet may provide an explanation for the situation just described, in which fatal niacin deficiency was produced in adult dogs on a diet containing 22 per cent casein and no corn meal. This recalls the failure of Goldberger and Tanner to treat successfully persons with pellagra with as much as 90 Gm. of casein per day.<sup>21</sup>

Since the "yield" of niacin derivatives in rat urine after tryptophane administration is quite low on a percentage basis, the possible conversion of tryptophane to niacin can be established best by a tracer experiment involving the administration of tryptophane labeled with isotopic carbon or nitrogen. The work of Krehl and associates<sup>22</sup> offers an alternative, albeit not mutually exclusive, mechanism for the tryptophane-niacin relationship. They observed

that niacin administration increased the efficiency of tryptophane utilization on a corn grits-low casein diet from 30 to 70 per cent. Similar studies with pyridoxine would probably yield a qualitatively similar result. Indeed, such a relationship might be demonstrable between each member of the B complex and the essential amino acids, although the present instance may operate via a specific metabolic path.

Of potentially great significance in this connection and elsewhere is the observation<sup>23</sup> that, like corn meal, the addition of gelatin or a tryptophane-free hydrolysate of fibrin to a low casein (9 per cent) ration markedly depressed the growth of rats and this could be prevented by the further addition of niacin or tryptophane. One explanation which suggests itself is that by improving the supply of other essential amino acids the relative deficiency in tryptophane is more marked and that such a situation is more deleterious to the animal's well-being than the original state of affairs. However, this would not account for the action of niacin. It may be that the increased over-all protein metabolism increases the demand for niacin much as an increased carbohydrate metabolism increases the demand for thiamine. There are, of course, other analogies to this situation, but it certainly suggests that an evaluation of the factors leading to the development of pellagra must consider not only the tryptophane content of the diet but the total amount of protein as well. That corn protein is tryptophane deficient has long been recognized and has been considered as a possible etiologic factor in pellagra by many investigators including Wilson, Mitchell, and Goldberger. However, no suggestion of a specific protein-tryptophane-vitamin P-P relationship was made in the literature until the recent work of the Wisconsin laboratory.<sup>10, 11</sup> Further investigation of the metabolic interrelationships of pyridoxine, tryptophane, niacin, and dietary protein is essential to the establishment of a rational concept of the etiology and pathogenesis of pellagra.

Complex as these interrelationships now seem, to the situation must be added the existence of toxic principles in corn and possibly other cereals. While the older literature contains many such speculations, no such material had been isolated until 1933 when Stockman and Johnston<sup>24</sup> reported that they had obtained from corn feed meal (corn from which the starch and oil had been removed) an acid which gave an insoluble lead salt and an easily crystallizable and soluble sodium salt. No attempt to establish the identity of this substance was made. Single doses of about 1 Gm. given to Rhesus monkeys resulted in drowsiness and mild paresis. Repeated doses produced drowsiness, general paresis, paralysis, coma, and death. On histologic examination the only significant lesions were found in the nervous system. They consisted largely of degenerative changes not unlike those reported from time to time as occurring in persons with pellagra, and more recently in dogs.<sup>25</sup> Guinea pigs responded in the same fashion but rabbits seemed resistant to the substance unless very large doses were employed, in which event they died after a few hours. It is unfortunate that no studies were made with dogs. A similar material was obtained from oats and from one batch of rye but not another. Considering the symptomatology reported, it is interesting to speculate upon the possible relationship of this material to the toxic principles responsible for favism, lathyrism, and "non-

convulsive ergotism" (which seems to be related to the ingestion of large quantities of rye bread uncontaminated with ergot) and the therapeutic and prophylactic use of niacin in these states.

Woolley<sup>26</sup> has presented a preliminary report in which is described the isolation from corn meal of an organic base, perhaps a pyridine derivative, which inhibited the growth of mice when added in small concentration to a synthetic ration. Such a substance may behave as a niacin (or, perhaps specifically, niacinamide) antagonist in the manner already described for beta-acetopyridine.<sup>27</sup>

A most recent pertinent report in this regard is that of Kodicek and associates<sup>28</sup> who observed that the inclusion of 1.5 mg. per cent of indole-3-acetic acid (the plant hormone, heteroauxin) in a 10.5 per cent casein diet suppressed rat growth, and this could be prevented by niacin or tryptophane fed at 1 and 20 mg. per cent, respectively. Since the corn meal content of indoleacetic acid has been estimated variously as between 20 and 100 mg. per kilogram,<sup>29, 30</sup> it seems possible that this may be the long sought, toxic principle of corn. Further, since both the auxins and the corn toxin of Stockman and Johnston was obtained by initial heavy metal precipitation, it may be that these authors obtained a crude preparation of indoleacetic acid thirteen years ago, although they failed to identify it as such. Woolley's pellagrenic agent is an organic base which can be extracted from an alkaline medium with chloroform and so cannot be indoleacetic acid, although the possibility remains that it is a basic precursor or bound form of heteroauxin.

The excretion of indole derivatives in pellagrous urine has long been known. These have included indican<sup>32, 33</sup> and indolethylamine.<sup>34</sup> While indoleacetic acid itself has not been isolated, it is known that the red pigment observed in the urine of persons with pellagra and blacktongue dogs, thought to be a porphyrin by Beckh and co-workers,<sup>35</sup> is a mixture of urorosein and a substance similar to indirubin.<sup>36, 37</sup> While nothing is known of the indirubin-like pigment, save for its indole nucleus, urorosein is actually excreted as uroroseinogen which was shown by Herter<sup>38</sup> to be identical with indoleacetic acid which is converted to the red pigment urorosein on standing in strongly acid solutions in the presence of traces of nitrite or other oxidizing agents.

Until recently we had considered the presence of these indole compounds the result of unusually active bacterial putrefaction in the bowel due to the serious disturbances of gastrointestinal function occasioned by pellagra. The demonstration of a tryptophane-nicotinic acid relationship<sup>10, 11, 13, 14, 22, 23</sup> then seemed to indicate a revision of this concept, although the presence of excessive quantities of indole derivatives in the urine of subjects on a purportedly tryptophane-deficient diet appeared irrational. The presence of such large quantities of heteroauxin in corn meal renders such reasoning unnecessary. However, it should be realized that the urine of normal rats on a synthetic diet gives a positive urorosein reaction roughly proportional to the level of dietary casein and greatly intensified by feeding extra casein or tryptophane.<sup>33</sup> If this reaction is due solely to indoleacetic acid (no other known derivative of trypto-



phane gives this reaction), this substance which cannot be further metabolized<sup>28</sup> appears to be a normal product of mammalian tryptophane metabolism.

Much work remains to be done to establish the mode of action of indoleacetic acid in order to determine its role in the etiology of pellagra. On purely structural grounds one might conceive readily of it as an inhibitor of the metabolism of either nicotinic acid or tryptophane or both. Does it block the conversion of tryptophane to niacin, inhibit coenzyme synthesis from niacin or niacinamide, or the conversion of niacin to niacinamide or vice versa, or perhaps stimulate the metabolism of mammalian cells as it does certain plant cells and thereby increase the niacin requirement as previously postulated for the effect of administration of tryptophane-deficient proteins? This might be clarified by studies with synthetic plant hormones (such as, 2,4 dichlorophenoxyacetic acid) which contain no indole nucleus. Dubos<sup>27</sup> has reported that indoleacetic acid and a number of related substances inhibit the growth of *Mycobacterium tuberculosis*, *Streptococcus hemolyticus*, *Streptococcus salivarius*, and *Shigella paradysenteriae*, and this inhibition of growth can be reversed by tryptophane in a concentration about ten times that of the inhibiting auxin. If this phenomenon represents a structural inhibition similar to those of the vitamin analogues studied by Woolley, it is most unusual to find the inhibitor functioning at a lower concentration than the normal metabolite and suggests a more specific role for the auxins. It is unfortunate that no study was made of nicotinic acid in this connection. On the other hand, in similar studies in this laboratory<sup>30</sup> we have not observed any inhibition of growth or acid production by indoleacetic acid in cultures of such variegated organisms as *Streptococcus faecalis*, *Lactobacillus arabinosis*, *Staphylococcus aureus*, *Proteus vulgaris*, *Aerobacter aerogenes*, and *Escherichia coli*, except when rather high concentrations of indoleacetic acid were employed. Then, however, the inhibition was not alleviated by niacin or tryptophane. These facts, coupled with the failure of indoleacetic acid to stunt the growth of almost half the rats in the study of Kodicek and associates,<sup>28</sup> warrant considerable caution in evaluating the role of this compound in the etiology of pellagra.

The final thread in this story, to date, is the observation of Raska<sup>31</sup> that administration of 0.4 Gm. of adenine plus sodium phosphate per day to dogs on a good, standard laboratory ration induced the development of a blacktongue-like syndrome accompanied by a moderate hypertension in as little as one week, the animal dying after fourteen days. Until this observation is coupled with a therapeutic or prophylactic trial of niacin, tryptophane, or even whole liver substance and until some data are available concerning the quantitative distribution of adenine in foodstuffs, it will not be possible to determine the significance of this observation in the etiology of pellagra. However, this finding well may be of far-reaching fundamental significance, and further investigation should certainly be prosecuted vigorously.

Therefore, it appears that pellagra, as it occurs endemic among a corn-eating population, is a disease involving the metabolic interrelationships of niacin, pyridoxine, tryptophane, indoleacetic acid, perhaps adenine, and at least one other unidentified toxic substance, to say nothing of the effects of the numerous other vitamin and amino acid deficiencies and general level of protein intake

associated with such a diet. Which, if any, of these factors other than the niacin is involved in the development of the fatal acidosis and dehydration of the crises of blacktongue and pellagra, as seen on a corn diet and the mode of action of niacin in this connection, remains obscure. Despite these many gaps in our comprehension of the pathogenesis of pellagra, to Harriette Chick<sup>40</sup> must go credit for what now seems the first proper formulation of pellagra; she states that "Pellagra is caused by a toxic substance derived from the maize diet, which can be corrected by sufficient 'good' protein or perhaps by sufficient vitamin B<sub>2</sub> (which is found to accompany the 'good' proteins)."

That corn is not essential to the pathogenesis of classical pellagra long has been known and has been described in epidemic proportions among the inhabitants of a Japanese prison camp<sup>41</sup> who were living on a diet high in carbohydrate (on a percentage basis) but low in calories, protein, sources of the B complex and entirely lacking animal protein. The pellagra was complicated by nutritional edema and beriberi, in contradistinction to the dehydration of pellagra seen among corn eaters, and consisted mainly of extensive lesions of the skin and the mucosa of the gastrointestinal tract. In this connection it is well to note that there exists, as yet, no correlation between the known physiologic and chemical mechanisms involving niacin and its derivatives and the integrity of the skin and mucosa. Nor is there any explanation for the observation that exposure of the skin to sunlight not only results in an exacerbation of the dermatitis but of the systemic manifestations of pellagra as well.<sup>42</sup> The pathogenesis of the Chittenden-Underhill syndrome<sup>43, 44</sup> needs further study. This syndrome which occurs in vitamin A-deficient pups may actually bear no etiologic relation to blacktongue and may be simply another expression of the disturbed "integrity" of mucosal and epithelial structures usually found in vitamin A-deficient animals. In this case it is manifest as an infection of the mouth with Vincent's organisms as also has been found in niacin deficiency blacktongue<sup>45</sup> but, perhaps for entirely different reasons.

A few other unsolved problems of niacin metabolism also may be worth mentioning. No explanation is available for the fact that niacin administration results in an elevated red cell concentration of the pyridine nucleotides while nicotinamide administration does not.<sup>46, 47</sup> This seems even more puzzling, considering the vitamin action of nicotinamide, the existence of nicotinamide and not the acid in the coenzyme molecules, and the fact that the tissue (including erythrocytes) enzyme which destroys these nucleotides does so by cleaving nicotinamide as such from the remainder of the molecule,<sup>48</sup> a reaction which is inhibited by the presence of nicotinamide but not niacin.<sup>49, 50</sup> In contrast, it is nicotinamide which has been reported to prevent cobalt-induced polycythemia in rabbits.<sup>50</sup> No information is available concerning the effects of niacin in this connection nor of the effects of niacin, niacinamide, cobalt, or combinations thereof on the pyridine nucleotide concentrations of red cells in this species. While niacin and niacinamide distribute themselves equally between erythrocyte and plasma water,<sup>47</sup> red cells show a considerable avidity for N<sup>1</sup>-methylnicotinamide, the erythrocyte concentration of the latter remaining considerably

elevated long after the plasma concentration has returned to normal following intravenous administration.<sup>51</sup>

Najjar and associates<sup>53</sup> have proposed that it is the methylated pyridinium form of niacin which may be the physiologically active form of the vitamin. They claim to have demonstrated antiblacktongue activity for N<sup>1</sup>-methylnicotinamide in dogs and also to have treated successfully a hamster, moribund in nicotinic acid deficiency.<sup>60</sup> (It may be noted parenthetically that other workers have failed thus far to produce nicotinic acid deficiency in the hamster.) This hypothesis was furthered by the report of Vance<sup>54</sup> describing amelioration of dermatitis and glossitis in a case of pellagra on treatment with N<sup>1</sup>-methylnicotinamide. However, since the psychomotor disturbances in this patient apparently became even more severe after this treatment and since the patient had previously been given 20 mg. of nicotinamide, it is not possible to evaluate properly this report. More recently, Najjar and Deal<sup>58</sup> have claimed to demonstrate demethylation of N<sup>1</sup>-methylnicotinamide by estimating its lipotropic activity in choline-deficient rats. However, the variable activity they obtained undoubtedly can be ascribed more properly to the diminished appetite of such rats; this, indeed, has been found to be the case in this laboratory.<sup>59</sup> The minute excretion of nicotinamide and nicotinic acid in the urine of these rats after eating diets containing 1 and 2 per cent of N<sup>1</sup>-methylnicotinamide easily may have been due to impurities in the N<sup>1</sup>-methylnicotinamide preparation. At any rate, useful demethylation does not appear to be a major metabolic pathway for this compound. Since this substance possessed no antiblacktongue activity when tested in the Wisconsin laboratory,<sup>55</sup> there is, as yet, little reason to believe that the methylated form of nicotinamide is the biologically active structure, although the methylation of nicotinamide yet may be found to serve a physiologically "useful" role. Further work also is required to elucidate the physiologic significance of the oxidation of N<sup>1</sup>-methylnicotinamide to the corresponding 6-pyridone.<sup>52</sup> No physiologic role has been ascribed to unbound niacin (that is, niacin not present as part of the pyridine nucleotides) which has been found in significant concentration only in the liver and whose concentration does not drop markedly even in animals dying of niacin deficiency, while that of the pyridine nucleotides is drastically reduced. This problem may be resolved by more accurate determinations of pyridine nucleotides in fresh liver.

#### REFERENCES

1. Elvehjem, C. A., Madden, R. J., Strong, F. M., and Woolley, D. W.: The Isolation and Identification of the Antiblacktongue Factor, *J. Biol. Chem.* 123: 137, 1938.
2. Goldberger, J., and Wheeler, G. A.: Experimental Blacktongue of Dogs and Its Relation to Pellagra, *Pub. Health Rep.* 43: 172, 1928.
3. Warburg, O., and Christian, W.: Co-Fermentproblem, *Biochem. Ztschr.* 275: 464, 1935.
4. von Euler, H., Albers, H., and Schlenk, F.: Ueber die Co-zy-mase, *Ztschr. f. physiol. Chem.* 237: 1, 1935.
5. Anonymous: Etiology of Blacktongue, *Nutrition Rev.* 1: 368, 1943.
6. Handler, P., and Dann, W. J.: The Biochemical Defect in Nicotinic Acid Deficiency, *J. Biol. Chem.* 145: 145, 1942.
7. Handler, P., and Featherston, W.: The Biochemical Defect in Nicotinic Acid Deficiency. II. On the Nature of the Anemia, *J. Biol. Chem.* 151: 395, 1943.
8. Handler, P.: Use of Highly Purified Rations in the Study of Nicotinic Acid Deficiency, *Proc. Soc. Exper. Biol. & Med.* 52: 263, 1943.

9. Aykroyd, W. R., and Swaminathan, M.: The Nicotinic-Acid Content of Cereals and Pellagra, *Indian J. M. Research* 27: 667, 1940.
10. Krehl, W. A., Teply, L. J., and Elvehjem, C. A.: Corn as an Etiological Factor in the Production of a Nicotinic Acid Deficiency in the Rat, *Science* 101: 283, 1945.
11. Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A.: Growth-Retarding Effect of Corn in Nicotinic Acid-Low Rations and Its Counteraction by Tryptophane, *Science* 101: 489, 1945.
12. Schaeffer, A. D., McKibbin, J. M., and Elvehjem, C. A.: Studies of the Vitamin B Complex in the Nutrition of the Dog, *J. Nutrition* 23: 491, 1942.
13. Rosen, F., Huff, J. W., and Perlzweig, W. A.: The Effect of Tryptophane on the Synthesis of Nicotinic Acid in the Rat, *J. Biol. Chem.* 163: 343, 1946.
14. Rosen, F., and Perlzweig, W. A.: Personal communication.
15. Lepkovsky, S., Roboz, E., and Haagen-Smit, A. J.: Xanthurenic Acid and Its Role in the Tryptophane Metabolism of Pyridoxine-Deficient Rats, *J. Biol. Chem.* 149: 195, 1943.
16. Umbreit, W. W., and Gunsalus, I. C.: The Function of Pyridoxine Derivatives: Arginine and Glutamic Acid Decarboxylases, *J. Biol. Chem.* 159: 333, 1945.
17. Schlenk, F., and Snell, E. E.: Vitamin B<sub>6</sub> and Transamination, *J. Biol. Chem.* 157: 425, 1945.
18. Najjar, V. A., and Wood, R. W.: Presence of a Hitherto Unrecognized Nicotinic Acid in Human Urine, *Proc. Soc. Exper. Biol. & Med.* 44: 386, 1940.
19. Huff, J. W., and Perlzweig, W. A.: A Product of Oxidative Metabolism of Pyridoxine, 2-Methyl-3-Hydroxy-4-Carboxy-5-Hydroxymethylpyridine (4-Pyridoxic Acid). I. Isolation From Urine. Structure, and Synthesis, *J. Biol. Chem.* 155: 345, 1944.
20. Singal, S. A., Briggs, A. P., Sydenstricker, V. P., and Littlejohn, J. M.: The Effect of Tryptophane on the Urinary Excretion of Nicotinic Acid in Rats, *J. Biol. Chem.* 166: 573, 1946.
21. Goldberger, J., and Tanner, W. F.: The Pellagra-Preventive Action of Dried Beans, Casein, Dried Milk, and Brewers' Yeast, With a Consideration of the Essential Preventive Factors Involved, *Pub. Health Rep.* 40: 54, 1925.
22. Krehl, W. A., de la Huerga, J., and Elvehjem, C. A.: The Effect of Niacin on the Utilization of Tryptophane, *J. Biol. Chem.* 164: 551, 1946.
23. Krehl, W. A., Sarma, P. S., and Elvehjem, C. A.: The Effect of Protein on the Nicotinic Acid and Tryptophane Requirement of the Growing Rat, *J. Biol. Chem.* 162: 403, 1946.
24. Stockman, R., and Johnston, J. M.: Cereal Food Poisoning and Its Relation to the Etiology of Pellagra, *J. Hyg.* 33: 204, 1933.
25. Jensenius, H., and Nogaard, F.: Studies on the Effect of a Pellagra-Producing Diet in Dogs, With Special Reference to the Histological Changes in the Central Nervous System, *Acta path. et microbiol. Scandinav.* 19: 433, 1942.
26. Woolley, D. W.: The Occurrence of a Pellagrigenic Agent in Corn, *J. Biol. Chem.* 163: 773, 1946.
27. Woolley, D. W.: Reversal by Tryptophane of the Biological Effects of 3-Acetylpyridine, *J. Biol. Chem.* 162: 179, 1946.
28. Kodicek, E., Carpenter, K. J., and Harris, L. J.: Pellagrigenic Activity of Indole-3-Acetic Acid in the Rat, *Lancet* 2: 491, 1946.
29. Haagen-Smit, A. J., Leech, W. D., and Bergren, W. R.: The Estimation Isolation and Identification of Auxins in Plant Materials, *Am. J. Botany* 29: 500, 1942.
30. Berger, J., and Avery, G. S.: Isolation of an Auxin Precursor and an Auxin (Indoleacetic Acid) From Maize, *Am. J. Botany* 31: 199, 1944.
31. Raska, S. B.: The Production of Experimental Pellagra by Adenine, *Science* 105: 126, 1947.
32. Hunter, A., Givens, M. H., and Lewis, R. C.: Pellagra, *Metabolic Studies*, Hygienic Lab. Bull. 102: 39, 1916.
33. Billings, F., Green, J. L., Ormsby, O. S., Webster, G. W., Grindley, H. S., Ricketts, J. T., Singer, A. D., and McNeal, W. J.: Pellagra in Illinois, *Arch. Int. Med.* 10: 123, 219, 1912.
34. Sullivan, M. X.: Indolethylamine in the Urine of Pellagrins, *J. Biol. Chem.* 50: XXXIX, 1922.
35. Beckh, W., Ellinger, P., and Spies, T. D.: Porphyrinuria in Pellagra, *Quart. J. Med.* 6: 305, 1937.
36. Watson, C. J., and Layne, J. A.: Studies of Urinary Pigments in Pellagra and Other Pathological States. I. Clinical Observations, *Ann. Int. Med.* 19: 183, 1943.
37. Schwartz, S., Marvin, J. F., Layne, J. A., and Watson, C. J.: Studies of Urinary Pigments in Pellagra and Other Pathological States. III. Certain Toluene Soluble Pigments of Human and Canine Urine, *Ann. Int. Med.* 19: 206, 1943.
38. Herter, C. A.: On Indolacetic Acid as the Chromogen of the Urorosein of the Urine, *J. Biol. Chem.* 4: 253, 1908.

39. Handler, P., and Kamin, H.: Unpublished observations.
40. Chick, H.: Current Theories of the Aetiology of Pellagra (De Lamar Lecture), *Lancet* 2: 341, 1933.
41. Lewis, C. F., and Musselman, M. F.: Observation on Pellagra in American Prisoners of War in the Philippines, *J. Nutrition* 32: 549, 1948.
42. Smith, D. T., and Rufin, J. M.: Effect of Sunlight on the Clinical Manifestations of Pellagra, *Arch. Int. Med.* 59: 631, 1937.
43. Chittenden, R. H., and Underhill, F. P.: The Production in Dogs of a Pathological Condition Which Closely Resembles Human Pellagra, *Am. J. Physiol.* 44: 13, 1917.
44. Underhill, F. P., and Mendel, L. B.: A Dietary Deficiency Canine Disease; Further Experiments on the Diseased Condition in Dogs Described as Pellagra-Like by Chittenden and Underhill and Possibly Related to So-called Blacktongue, *Am. J. Physiol.* 83: 589, 1928.
45. Smith, D. T., Persons, E. L., and Harvey, H. I.: On the Identity of the Goldberger and Underhill Types of Canine Blacktongue. Secondary Fuso-Spirochetal Infection in Each, *J. Nutrition* 14: 373, 1937.
46. Hoagland, C. L., and Ward, S. M.: The Quantitative Determination of Factor V by Measurement of Nitrite Produced by Hemophilus Influenzae, *J. Biol. Chem.* 146: 115, 1942.
47. Handler, P., and Kohn, H. I.: The Mechanism of Cozymase Synthesis in the Human Erythrocyte; a comparison of the Roles of Nicotinic Acid and Nicotinamide, *J. Biol. Chem.* 150: 447, 1943.
48. Handler, P., and Klein, J. R.: The Inactivation of Pyridine Nucleotides by Animal Tissues in Vitro, *J. Biol. Chem.* 143: 49, 1942.
49. Mann, P. J. G., and Quastel, J. H.: Nicotinamide, Cozymase and Tissue Metabolism, *Biochem. J.* 35: 502, 1941.
50. Göbell, O., and Lang, H.: Die Wirkung des Nicotinsäureamids auf die durch Kobalt-sulfat erzeugte Polyglobulie beim Kaninchen, *Arch. f. exper. Path. u. Pharmacol.* 198: 70, 1941.
51. Perlzweig, W. A.: Personal communication.
52. Knox, W. E., and Grossman, W. I.: A New Metabolite of Nicotinamide, *J. Biol. Chem.* 166: 391, 1946.
53. Najjar, V. A., Hammond, M. M., English, M. A., Wooden, M. B., and Deal, C. C.: The Anti-Blacktongue Activity of N-Methylnicotinamide Chloride, *Bull. Johns Hopkins Hosp.* 74: 406, 1944.
54. Vance, G. A.: Clinical and Electrocardiographic Response to N-Methylnicotinamide Chloride Administration in Man, *Bull. Johns Hopkins Hosp.* 77: 393, 1945.
55. Teply, L. J., Krehl, W. A., and Elvehjem, C. A.: Biological Activity of N-Methylnicotinamide and Nipicotic Acid, *Proc. Soc. Exper. Biol. & Med.* 58: 169, 1945.
56. Handler, P., and Dubin, I. N.: The Significance of Fatty Infiltration in the Development of Hepatic Cirrhosis Due to Choline Deficiency, *J. Nutrition* 31: 141, 1946.
57. Dubos, R.: Inhibition of Bacterial Growth by Auxins, *Proc. Soc. Exper. Biol. & Med.* 63: 317, 1946.
58. Najjar, V. A., and Deal, C. C.: The Demethylation of N<sup>1</sup>-Methylnicotinamide and the Influence of the Methyl Group on the Fatty Liver of Rats, *J. Biol. Chem.* 162: 741, 1946.
59. Briggs, G. M., Groschke, A. C., and Lillie, R. J.: Effect of Proteins Low in Tryptophane on Growth of Chickens and on Laying Hens Receiving Nicotinic Acid-Low Rations, *J. Nutrition* 32: 659, 1946.
60. Najjar, V. A., Scott, D. B. M., and Holt, L. E., Jr.: Observations on the Nature and Properties of the Fluorescent Factor F<sub>2</sub>, *Science* 97: 537, 1943.

## NITROGEN BALANCE

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**S**TUDIES of nitrogen balance have been made in countless physiologic and pathologic conditions. The scheme given herein, while obviously inadequate, is offered as a framework for the consideration and interpretation of such data.

The proteins referred to are the tissue proteins in general. While one might consider the liver proteins to be most important, it has been amply demonstrated that in hemorrhagic shock there occurs a liberation of amino acids from the proteins of skeletal muscle.<sup>1</sup> The processes involved in reactions 1 and 2 are but little understood. Nevertheless, the classical work of Schoenheimer and colleagues<sup>2</sup> has left no doubt that this interchange is of a rapid, dynamic, and constant nature heretofore totally unrealized. There must exist a host of factors which influence the rate, extent, and equilibrium point of such reactions. Reason does exist to suspect that these reactions do not necessarily entail complete degradation and resynthesis of the tissue protein molecules. A deficiency of a single essential amino acid, even in the adult expected to be in nitrogen equilibrium, would suffice to maintain the equilibrium in favor of reaction 2 since there is no reason, at present, to suspect the synthesis of a protein molecule lacking any one of its normal constituent amino acids. Interruption of endocrine control (the anterior pituitary) perhaps may shift the reaction toward amino acid accumulation as may also excessive adrenocortical or thyroid activity, while excessive pituitary function and testosterone administration in both sexes increase nitrogen retention and protein accumulation. Since the reactions involved in protein synthesis are endergonic it should be expected that any disturbance of intracellular energy metabolism, the simplest being anoxia, would also interfere with protein synthesis and result in accumulation of amino acids. All told, the mechanisms involved in reactions 1 and 2 and the physiologic factors governing them await considerable investigation.

Reaction 3 represents the reversible formation and degradation of non-protein materials. There are only a few known examples of this type, such as glutathione, and they may be ignored in the remaining discussion. Reactions 4 and 5 represent the reversible oxidative deamination and transamination of amino acids. Again, the exceedingly rapid and constant rate of these reactions has been demonstrated by Schoenheimer and colleagues. However, the succeeding steps, urea formation (reaction 6) and ultimate CO<sub>2</sub> production (reaction 14), are not reversible. Any process which accelerates either of these reactions will automatically accelerate the other as reaction 5 then is no longer possible. The increased demands for energy or glucose formation imposed by such conditions as total inanition, partial caloric starvation, and diabetes may be regarded as examples of negative nitrogen balance due to acceleration of reaction 11. These

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conditions may be under nervous or endocrine control or may be simply the result of driving a reaction "to the right" by removing one of the end products. No circumstance is known in which one can definitely attribute negative nitrogen balance to increased urea synthesis, although this remains a possibility in some forms of negative nitrogen balance resulting from trauma, and in fasted animals after administration of adrenocortical extract. However, were such a situation to arise it could, perhaps, be demonstrated and alleviated temporarily by administration of purine drugs (caffeine, theobromine) which inhibit urea formation by way of the ornithine cycle but not the glutamine mechanism.<sup>5</sup>

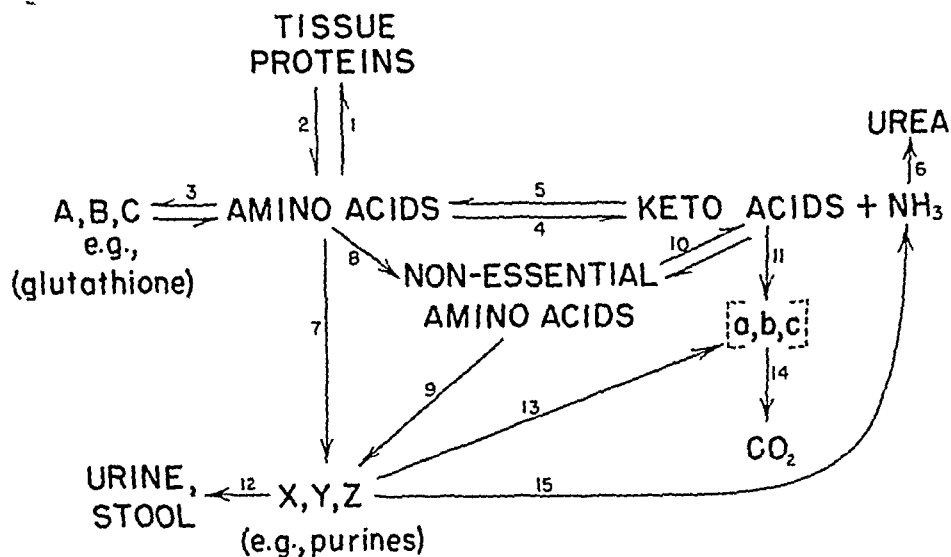


Fig. 1.—The over-all outlines of nitrogen metabolism.

Reaction 7 is the irreversible formation of nitrogenous derivatives of amino acids, that is, melanin, adrenalin, histamine, creatine, sarcosine, choline,  $\beta$ -alanine, carnitine, pyrrol compounds, purines, pyrimidines. It may be assumed that when these substances are formed the carbon skeletons of the essential amino acids from which they are derived are irretrievably lost for purposes of protein synthesis. In the same fashion the synthesis of nonessential amino acids from essential precursors (cystine from methionine, tyrosine from phenylalanine), reaction 8, also results in the irreversible loss of the essential amino acids involved. The apparent resynthesis of essential amino acids from nonessentials via reactions 8, 10, and 5 does not occur since, of course, entirely different  $\alpha$ -keto acids are involved.

While little is known of the factors involved in positive nitrogen balance, there seems little reason to believe that it is the consequence of the complex phenomena necessary to dam reactions 4, 7, 8, etc. It would seem more reasonable to consider positive balance the result of a displaced equilibrium of reactions 1 and 2, perhaps with the growth hormone of the anterior pituitary as the dominant controlling factor. Again, no definite evidence is available

to decide whether this operates by decelerating protein hydrolysis (reaction 2) or accelerating its synthesis (reaction 1). However, the studies of Schoenheimer suggest that consideration is necessary only of altered reaction rates as both processes are in constant flux in young growing animals as they are in adults.

Negative nitrogen balance has been observed in many different circumstances. The situation to be considered first is that in which an adult subject is offered a diet more than adequate in all known respects but completely lacking protein or amino acids. The nitrogen excretion in adult human beings under such circumstances has been variously estimated at .025 to .04 Gm. per kilogram per day (2 to 4 Gm. per adult) of which a relatively small fraction is urea nitrogen. This value, then, represents the daily obligatory nitrogen loss and is the resultant total of all the obligatory irreversible reactions involved in the previously mentioned scheme. It is identical with what Folin termed endogenous nitrogen, although the endogenous-exogenous concept is invalid under all other circumstances.<sup>2</sup> It is not possible to determine to what extent the urea excretion is simply the consequence of reactions 4 and 6 or whether it is largely due to reactions 7, 8, 9, etc. The low rate of urea excretion suggests that the latter possibility is the more likely.

The second situation is the negative nitrogen balance associated with a deficiency of a single essential amino acid. It is obvious that in a serious deficiency of this sort no new protein synthesis is possible, as incomplete proteins are unlikely, and the bulk of the dietary nitrogen so provided would be discarded in the urine in various forms but largely as urea. However, there then still exists an obligatory metabolism of the missing amino acid. It is readily available from reaction 2. Some of that amino acid must then be metabolized via the paths of reactions 4, 7, 8, and 11. But, as a result, the other amino acids originally present in the protein from which this "catabolized" amino acid was derived are no longer useful for protein synthesis and are in consequence degraded and discarded. The extent of negative nitrogen balance is, therefore, far greater than that due to the loss of the nitrogen in the missing amino acid alone.

Were this description of the situation complete, negative nitrogen balance in single amino acid deficiencies should never be greater than that seen in the total protein deficiency previously discussed. In the latter situation the amount of nitrogen lost should be determined by that amino acid whose obligatory metabolism is greatest. Such data are not available yet for the ten possible deficiencies in order to determine which acid is the limiting factor, and this will be possible only when carefully controlled determinations are performed using the same subjects for all deficiencies. The same subjects also should undergo complete protein deficiency on an otherwise adequate diet, and in each case sufficient time must be allowed for the new equilibrium to be attained as the adjustment to a new "plane of protein nutrition" appears to be a rather slow process. However, reason does exist to suspect that the negative nitrogen loss amino acid deficiencies than in total protein depriva-



tion. While many other examples may exist in the literature, two may be taken from recent publications. Thus, it has been found that the addition of 6 per cent gelatin or a tryptophane-free fibrin hydrolysate to a diet containing 9 per cent casein actually markedly depressed the growth of young rats to a level below that on the basal 9 per cent casein diet.<sup>4</sup> Growth was restored to its original level by the further addition of tryptophane (or nicotinic acid), seemingly indicating that an increased total amino acid ingestion also increased specifically the tryptophane or nicotinic acid requirement. The failure of tryptophane plus gelatin to increase the growth rate above that on the basal diet is taken to indicate another amino acid as the limiting growth factor. Earlier, Jackson and associates<sup>5</sup> considered the rat growth inhibition occasioned by the addition of gelatin to a high casein diet, the result of a toxic property of gelatin, since growth could not be restored by addition of the amino acids known to be lacking in gelatin. This does not appear to be the case in the work with fibrin hydrolysates and further work is required before the status of gelatin can be certain. Similarly, Kornberg,<sup>6</sup> investigating the nutritive requirements for granulocyte production in the rat, found that the addition of methionine and tryptophane to a basal gelatin diet actually increased the mortality rate from 25 to 90 per cent in a four-day test period. Reasoning from these two examples, then, it seems not unlikely that, in like fashion, single deficiencies may induce a greater nitrogen loss than that of total protein deprivation. If this proves to be true, no mechanism for the phenomenon is yet available. The situation, however, is analogous to that long known to investigators of B complex deficiencies, namely, that single vitamin deficiencies (such as thiamine) result in a more dramatic weight loss and earlier deaths than do diets devoid of all members of the B complex. The physiologic factors involved in such deficiencies may be elucidated by more complete investigation of the mechanisms involved in the toxicity of single amino acids, virtually all of which are lethal when fed or given parenterally in large quantities.<sup>21</sup>

Few quantitative data are available concerning the extent of nitrogen loss in human single amino acid deficiencies. Tryptophane and lysine deficiency resulted in a maximum daily negative balance of about 4 Gm. each of nitrogen,<sup>7</sup> while valine and methionine deficiencies resulted in maximum daily nitrogen losses of 2.9 and 1.6 Gm., respectively, per day.<sup>8</sup> No quantitative data were included in the report that deficiencies of threonine, leucine, isoleucine, and phenylalanine also result in negative nitrogen balance.<sup>9</sup> All these were tested on healthy adult male subjects on a diet providing approximately 3,000 calories and 7 Gm. of nitrogen per day. It is noteworthy that, thus far, no deficiency of a single amino acid has occasioned a nitrogen loss greater than that of total protein deprivation, although it should be added that these all have been relatively brief experiments. It will be of interest to learn the extent of nitrogen loss in the other possible deficiencies as well as the effect of increasing the total nitrogen intake. The failure of histidine deficiency to evoke a nitrogen loss under similar conditions in the same subjects<sup>9</sup> seems surprising in view of the high histidine content of globin and the rapid turnover of erythrocytes.

The third commonly observed situation involving negative nitrogen balance is that occasioned by various forms of trauma, such as burns, fractures, surgery, hemorrhage, and even complete bed rest. The nitrogen lost here is excreted largely as urea. (The loss through weeping exudates may be disregarded.) No critical data yet have been obtained to determine the underlying basis for this phenomenon, nor is there necessarily a common mechanism; however, until proved otherwise it is helpful to consider it of the same origin as the so-called toxic destruction of protein associated with acute infectious states. By and large, the nitrogen loss appears to be proportional to the severity of the trauma or infection although there have been numerous exceptions reported. These negative balances have been quite refractory to parenteral or dietary protein. Instead, the administration of the equivalent of about 15 Gm. of nitrogen (as protein or an amino acid mixture) has usually resulted in an almost equivalent increment in urinary nitrogen excretion. However, protein therapy has proved most beneficial during convalescence from such injuries.

Considering, then, the various possibilities in the scheme, reaction 2 seems a definite possibility in view of the marked increase in blood amino acids found in hemorrhagic shock.<sup>10</sup> This perhaps might be occasioned either by moderate tissue anoxia or direct adrenocortical control. It is noteworthy that anterior pituitary growth hormone does not prevent or diminish the negative nitrogen balance observed in rats with fractured femurs.<sup>11</sup> The possibility of tissue anoxia does not seem significant in subjects with bone fractures or those who were simply put to complete bed rest. However, here the atrophy of the *immobilized* limb cannot be disregarded, and whatever may control this situation, it cannot be stated whether the total negative nitrogen balance observed after fractures is any greater than that of the injured member itself, a phenomenon which also may occur on a small scale in all the skeletal muscle of the subject during complete bed rest.

Excessive urea production as the initial basis of negative nitrogen balance has not been demonstrated. If it does occur, it could be demonstrated by purine administration. When the latter depresses urea excretion, a rise in the blood concentration and urinary excretion of amino acids should be expected when excessive urea synthesis itself is not the primary malefactor. Nor has any great demand for glucose or fat synthesis after trauma been observed while the administration of glucose in febrile states has not diminished appreciably the nitrogen loss.

Reactions 7 and 8 remain untested and likely possibilities. Should trauma occasion an increased irreversible production of some substance, X, from an essential amino acid precursor, this would be tantamount to the production of a single amino acid deficiency. The extensive negative balances of burned subjects have been rather refractory to parenteral or dietary protein therapy. If negative balance were caused by such a physiologic rather than nutritional essential amino acid deficiency, then it might be presumed that equilibrium could not be restored by providing total protein equivalent to that lost as urea but only by meeting the unusual requirement for the particular amino acid in question.

Only one amino acid, methionine, has been so tested and it was found effective in reducing the negative balance of burned rats on a low protein diet by Croft and Peters.<sup>12</sup> Increasing the dietary protein level also prevented negative balance, even though the increased protein supplied only one-third as much methionine as the minimum effective supplement of free amino acids. The nitrogen loss in these animals was not as dramatic as that in burned human beings and also differed in that the situation provoking nitrogen loss in burned human beings is refractory to protein therapy for a considerable period after the accident. Moreover, this effect of methionine has not been confirmed in burned rats and human beings by other investigators<sup>13, 22</sup> including those in this laboratory.<sup>14</sup> Nevertheless, the theoretical significance and clinical potentialities of such therapy certainly warrant a complete investigation of this and the other essential amino acids in like fashion.

It is noteworthy also in this connection that those investigators who have sought the minimum level of protein intake which would maintain the adult in nitrogen balance usually have thought that cannibalism would be the ideal situation in that this would most nearly provide an amino acid mixture of the desired proportions. While this may yet prove true for the growing animal, it is not true necessarily of the adult since there is no evidence to indicate that the pattern of obligatory daily amino acid destruction represented by reactions 4, 6 to 9, and 11 to 14 in anywise resembles quantitatively the relative concentrations of the amino acids of the tissue proteins. For this reason, the relative proportions of the dietary amino acids for optimum nutrition in infants and children may bear little resemblance to adult maintenance requirements.

The nature and locus of the action of endocrine control of nitrogen metabolism are now entirely obscure. Certainly, further investigation of the role of the anterior pituitary, adrenal cortex, thyroid, and gonads is warranted. The possibility of nervous control has received comparatively little attention. A clue of great potential significance is offered by patients with brain injuries who have been observed by neurosurgeons to develop perforating gastric and duodenal ulcers within twenty-four hours of the brain accident. It has been well established that prolonged ingestion of low protein diets results in the development of gastric and duodenal ulcers in laboratory animals,<sup>15-19</sup> and Somervell<sup>20</sup> has called attention to the high incidence of duodenal ulcers in the population in the south of India which subsists on an extremely low protein ration. It seems possible, therefore, that such injured patients may develop a fulminating protein deficiency which in turn leads to the ulceration.\* Such ulcers are not uncommon in burned patients as well and liver damage in the latter also has been found, perhaps again indicating the possibility of an induced specific amino acid deficiency. This situation might also provide a bridge between the biochemical factors (gastric acidity, pepsin, protein deficiency), the physiologic factors (action of the vagus, pylorospasm), and the unquestionable psychogenic factors in ulcer formation.

\*This possibility arose in conversations with Dr. J. S. Harris, with whom studies of this situation are now in progress.

## REFERENCES

1. Russell, J. A., and Long, C. N. H.: Amino Nitrogen in Liver and Muscles of Rats in Shock After Hemorrhage, *Am. J. Physiol.* 147: 175, 1946.
2. Schoenheimer, R.: *The Dynamic State of Body Constituents*, Cambridge, 1942, Harvard University Press.
3. Bernheim, F., and Bernheim, M. L. C.: The Effect of Methyl Xanthines on the Formation of Urea in Rabbits, *Am. J. Physiol.* 147: 428, 1946.
4. Krehl, W. A., Sarma, P. S., and Elvehjem, C. A.: The Effect of Protein on the Nicotinic Acid and Tryptophane Requirement of the Growing Rat, *J. Biol. Chem.* 162: 403, 1946.
5. Jackson, R. W., Sommer, B. E., and Rose, W. C.: Experiments on the Nutritive Properties of Gelatin, *J. Biol. Chem.* 80: 167, 1928.
6. Kornberg, A.: Amino Acids in the Production of Granulocytes in Rats, *J. Biol. Chem.* 164: 203, 1946.
7. Holt, L. E., Jr., Albanese, A. A., Buemback, J. E., Jr., Kajdi, C., and Wangerin, D. M.: Nitrogen Balance in Experimental Tryptophane Deficiency in Man, *Proc. Soc. Exper. Biol. & Med.* 48: 726, 1941.
8. Rose, W. C., Haines, W. J., and Johnson, J. E.: The Role of the Amino Acids in Human Nutrition, *J. Biol. Chem.* 146: 683, 1942.
9. Rose, W. C., Haines, W. J., Johnson, J. E., Warner, D. T.: Further Experiments on the Role of the Amino Acids in Human Nutrition, *J. Biol. Chem.* 148: 457, 1943.
10. Wilhelmi, A. E., Russell, J. A., Engel, F. L., and Long, C. N. H.: Effects of Hepatic Anoxia on Respiration of Liver Slices in Vitro, *Am. J. Physiol.* 144: 669, 1945.
11. Bennett, L. B., Applegarth, A. P., and Li, C. H.: Effect of Anterior Pituitary Growth Hormone on Urinary Nitrogen Loss Following Fracture, *Proc. Soc. Exper. Biol. & Med.* 62: 42, 1946.
12. Croft, P. B., and Peters, R. A.: Nitrogen Loss After Thermal Burns; Effects of Adding Protein and Methionine to Diet of Rats, *Lancet* 1: 266, 1945.
13. Abbott, W. E., Hirshfield, J. W., Williams, H. H., Pilling, M. A., and Meyer, F. L.: Metabolic Alterations Following Thermal Burns. VI. The Effect of Altering the Nitrogen and Caloric Intake or of Administering Testosterone Propionate on the Nitrogen Balance, *Surgery* 20: 284, 1946.
14. Handler, P.: Unpublished data.
15. Hoelzel, F., and Da Costa, E.: Production of Pylorospasm and Prepyloric Ulcers in Rats, *Proc. Soc. Exper. Biol. & Med.* 29: 979, 1932.
16. Orten, A. V.: Some Observations on the Effects of Low Dietary Protein, *J. Nutrition* 17: 14, 1939.
17. Weech, A. A., and Paige, B. H.: Nutritional Edema in the Dog. IV. Peptic Ulcer Produced by the Same Low Protein Diet That Leads to Hypoproteinemia and Edema, *Am. J. Path.* 13: 249, 1937.
18. Fouts, P. J.: Vitamin B Complex Studies in Dogs; Production of Cirrhosis of Liver, *J. Nutrition* 25: 217, 1943.
19. Li, T. W., and Freeman, S.: The Frequency of "Peptic" Ulcers in Protein-Deficient Dogs, *Gastroenterology* 6: 140, 1945.
20. Somervell, T. H.: Further Contributions to the Causation and Treatment of Duodenal Ulcer and Its Complications, *Brit. J. Surg.* 30: 113, 1942.
21. Martin, G. J.: Toxicity of Amino Acids as Influenced by Riboflavin Deficiency, *Proc. Soc. Exper. Biol. & Med.* 63: 528, 1946.
22. Schenker, V.: The Characteristic Changes in Nitrogen Metabolism After Damage, *Proc. Canadian Physiol. Soc., Ninth Annual Meeting*, 1945.

# LABORATORY METHODS

## ASSAY OF PENICILLIN, STREPTOMYCIN, TRIVALENT ORGANIC ARSENICALS, AND OTHER BACTERICIDAL AND BACTERIOSTATIC AGENTS

A TECHNIQUE BASED ON AN ORIGINAL APPLICATION OF MATHEMATICAL PRINCIPLES  
WITH ADAPTATIONS FOR ALL ASSAY PURPOSES

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WITH THE TECHNICAL ASSISTANCE OF BARBARA GAMBLE, B.S.

A SIMPLE, rapid, accurate, flexible technique for assay of antibacterial agents has been described elsewhere.<sup>1</sup> In the same article nomographs are published permitting determination of results of the assay by inspection. Also in this article are given the details of the materials and equipment needed and references to previously published assay methods.

The present article is written for those investigators who wish a full understanding of the mathematical basis for the calculation, an outline of its adaptability for various purposes, an idea of the accuracy attainable by each of these adaptations, and an understanding of the effects of the various variables investigated in the course of developing the method.

Foster and Woodruff<sup>2</sup> reviewed the penicillin assay methods available and formulated certain criteria for an "ideal" method. While many assay methods have been published since the date of this excellent review, none have generally displaced those in use in 1943. The investigation herein described was initiated in an attempt to meet the requirements of these authors.

### THE PRINCIPLE OF THE METHOD

Our observations have shown that:

1. The growth of bacteria in culture of the type used in these experiments is directly proportional to the change in optical density of the culture.
2. The optical density can be read directly on the Klett-Summerson colorimeter (scale is  $= 500 \times$  optical density).
3. An accurate growth curve can be plotted using scale readings directly from the Klett-Summerson colorimeter (1 division  $= 20,000,000$  colonies per cubic centimeter).
4. The tangent of the angle between the straight-line growth curve of a control and of antibiotic-containing culture is directly proportional to the antibiotic concentration under the conditions specified.

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5. This tangent can be determined from the difference in turbidimetric readings which occurs with time in each culture during the straight-line phase of growth. Therefore, only one control and one known concentration of penicillin are needed to determine many unknowns, and a determination is possible before the difference in turbidimetric readings between the control and penicillin-containing cultures is statistically significant.

When, at a later time, the difference in turbidimetric readings between the control and penicillin-containing cultures is statistically significant, this difference is proportional to the square root of the penicillin concentration; therefore, the computation may be made by both methods if desired.

#### STANDARD TECHNIQUE

This permits assay by each of two methods on the same sample. All techniques are described for penicillin assay first, since only minor modifications are necessary for assay for streptomycin, trivalent organic arsenicals, or other antibiotics. All manipulations are made with sterile tuberculin or 5 c.c. Luer syringes and 26 gauge needles through 70 per cent alcohol on vaccine vial caps. The 0.85 per cent NaCl used for dilutions and the bactotryptose phosphate broth for the cultures are kept in sterile vaccine vial-capped containers. All photoelectric turbidimetric readings are made with the Klett-Summerson photoelectric colorimeter, using a No. 66 filter, on a 5 c.c. volume in the vaccine vial-capped colorimeter tubes for this instrument. All concentrations, unless otherwise specified, refer to the concentration per cubic centimeter in this 5 c.c. volume. The organism found most suitable for the test is the Oregon-J strain of *Staphylococcus aureus* obtainable from the American Type Culture Collection\* by request for No. 9801 or from the National Collection of Type Cultures† by request for No. 6982. After incubation until growth has reached the proper turbidity, keep the inoculated culture in an ice bath in the refrigerator. It is usable for a week.

The following photoelectric colorimeter readings are made:

- A = the broth blank on each new lot of bactotryptose broth
- B' = the culture blank on inoculated broth before placed in the ice bath; should be checked just before use each day
- B' - A = turbidity due to bacterial growth in the broth culture and should be 12 to 15; 15 corresponds to a colony count of 300,000,000 per cubic centimeter

Set up a control, a known, and as many unknown photoelectric colorimeter tubes as desired, as shown in Table I. The known should contain 0.04 unit of penicillin per cubic centimeter corresponding to the addition of 0.5 c.c. of a 0.4 unit per cubic centimeter dilution of a standard penicillin preparation. Concentrated unknowns should be diluted to an estimated concentration of 0.2 to 0.4 unit per cubic centimeter and 0.5 c.c. used. On each of these tubes in rapid sequence take the following readings:

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†The Lister Institute, London, England.

- B: at 0 time just before putting tubes in the 37 degree water bath;  
this is the culture blank  
C: 1.5 hours after incubation is started  
D: 2 hours after incubation is started  
E: 2.5 hours after incubation is started  
F: 4 to 30 hours after incubation is started, usually the next morning

Reading D may be omitted except when unusually high concentrations are anticipated or the earliest possible result is desired. Reading B is necessary only when unknowns differ widely in turbidity or color. During the interval between Readings B and C, the cultures are in the lag phase of growth, the growth curves are not straight line, and there is little antibiotic action.

$D - C = y_1$ , the turbidity due to bacterial growth during the first half hour of straight-line growth

$E - D = y_2$ , the turbidity due to bacterial growth during the second half hour of straight-line growth

Ideally,  $y_1$  and  $y_2$  should be equal.

$E - C = y_3$ , the turbidity due to bacterial growth in the first hour of straight-line growth,  $= y_1 + y_2$

The figure usually used in the calculation by Method 1 is  $y_3$ .

$F - B$  = turbidity due to bacterial growth called corrected F, and the difference between corrected F for the control and for each of the antibiotic-containing cultures is called  $y$ .

This is the figure used in calculation by Method 2. Data are recorded as shown in Table I.

TABLE I. DATA OF TYPICAL EXPERIMENT

		TUBE 1—CONTROL (C.C.)		TUBE 2—KNOWN (C.C.)		TUBE 3—UNKNOWN (C.C.)	
Add							
0.85% NaCl		0.5		—		—	
0.4 U. per c.c. Na penicillin		—		0.5		—	
Unknown solution		—		—		0.5	
Broth culture		4.5		4.5		4.5	
READING	TIME* (HR.)	COLOR- IMETER READING	DIFFER- ENCE Y	COLOR- IMETER READING	DIFFER- ENCE Y	COLOR- IMETER READING	DIFFER- ENCE Y
A=10, broth blank	0						
B, blank for F	0	20		21		20	
C	1.5	60		54		56	
$y_1 = D - C$			21		13		16
D	2.0	81		67		72	
$y_2 = E - D$			22		12		16
E	2.5	103		79		88	
$y_3 = E - C = y_1 + y_2$			43		25		32
F	22.5	230		51		110	
Corrected $F = (F - B)$		210		30		90	
$y_4 = F - F_3$ , or $y$					180		120

\*In the 37° C. water bath.

## THEORY OF THE CALCULATION

Note that area I in Fig. 1 shows the growth curves of a control and a penicillin-containing known or unknown of a typical experiment. Area II is an enlargement of the indicated portion of area I with a relative increase in scale

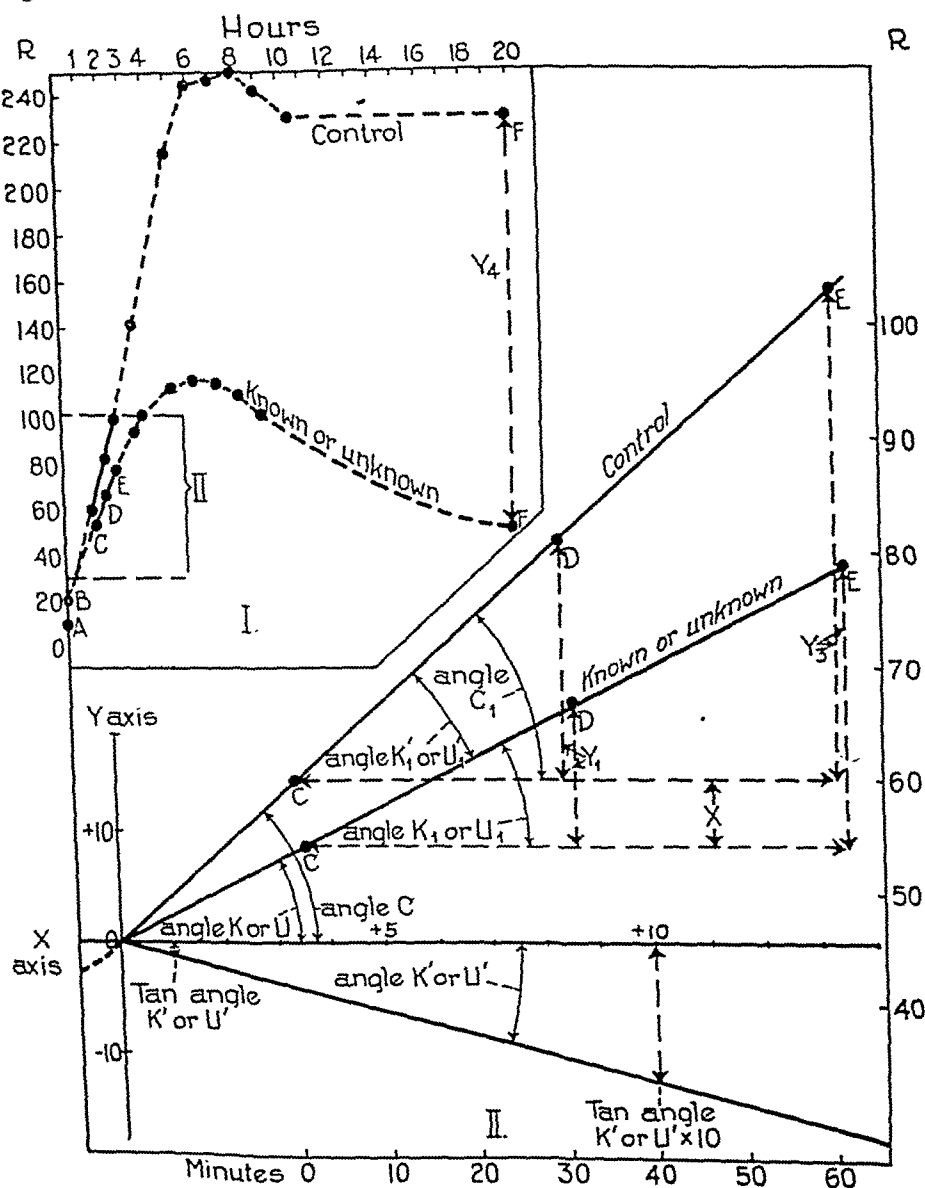


Fig. 1.—Explanation of the theory of the computation.

on the X axis to separate the curves for greater clarity. R refers to photoelectric colorimeter readings. The time scale for area I is in hours; for II it is in minutes, with the 0 beginning at the time of Reading C. In a typical experiment this is 1.5 hours after the tubes were placed in the 37° C. water bath, but



it may be any time while growth is straight line. A, B, C, D, E, and F are, respectively, the photoelectric colorimeter readings for the broth blank, the culture blank, the three readings during the 1-hour period used in the calculation for Method 1, and the final reading at 18 to 24 hours used in the calculation by Method 2. Note in the enlarged area II that the straight-line growth curves of the control and penicillin-containing known or unknown separate at the point marked 0, where the Y and X axis are drawn to intersect, and that prior to this, as indicated by the curved dotted line, the growth is not straight line and there is no difference in turbidity between the control and the penicillin-containing cultures. Note that  $y_1$  is the difference between the colorimeter reading, D, of the control or penicillin-containing culture and its reading, C, 30 minutes earlier, and that this forms one side of a right triangle with the time interval, X, of 30 minutes, or 5 units on the X axis forming the other side and the growth curve the hypotenuse. The same is true for the difference,  $y_3$ , between the Readings C and E, except that here the value of X is 1 hour or 10 units on the X axis. Therefore, the value of  $y_1$  divided by the corresponding value of X is the tangent of the angles  $C_1$  or of  $K_1$  or  $U_1$ . Since parallel lines intersecting a straight line form equal angles, angle  $C_1 = \text{angle } C$  and angle  $K_1$  or  $U_1 = \text{angle } K$  or  $U$ . By inspection it is evident that angle C is the angle which the control makes with the X axis and angle K or U is the angle which the penicillin-containing culture makes with the X axis. It is apparent that  $y_1$  or  $y_3$  divided by the corresponding values of X give the tangents of  $C_1$ ,  $K_1$ , or  $U_1$  and hence of C, K, or U. Note further that while the Readings C, D, and E are taken later for the penicillin-containing known or unknown than for the control, this time lag does not alter the value of the tangents so long as the growth curves are still in the straight-line phase. Note by inspection that angle C - angle K or U = angle  $K'_1$  or  $U'_1$ , the angle at which the penicillin-containing culture leaves the control culture. By construction, angle  $K'$  or  $U' = \text{angle } K'_1$  or  $U'_1$ . This construction is equivalent to making the growth curve of the control coincide with the X axis and rotating the growth curve of the penicillin-containing culture through an equal arc. The tangent of angle  $K'$  or  $U'$  then is a measure of the rate at which the growth curves of the antibiotic-containing cultures are deviating from the growth curve of the control culture.

This method of computation allows for differences in effectiveness of the antibiotic agents with differences in growth rate. It has been found, as shown by the results reported in Tables IV to VI for Method 1, that the tangent of the angle between the growth curves of the control and the known or unknown cultures is directly proportional to the antibiotic concentration for the antibacterial agents so far tested. Because it is so inconspicuous in the diagram at the point where  $x = +1$ , the numerical value of  $10 \times \tan K'$  or  $U'$  also is shown in Fig. 1 at the point where  $x = +10$ .

The calculation by Method 2 is based on the observation that the square root of the penicillin concentration is proportional to the difference between the photoelectric colorimeter readings due to turbidity from bacterial growth of the control culture and the penicillin-containing culture after 4 to 6 hours of incubation. Since this difference increases with time, computations by this method

increase in accuracy as long as the difference increases. Computation by Method 2 may be useful at 3 to 6 hours if inadvertently a higher concentration of penicillin was introduced than intended and the  $y_1$  readings were already beyond the straight-line phase.

## CALCULATION

An example of the calculation is given in Table II.

*Method 1.*—Let  $x$  = time in 0.1 of an hour or 6-minute intervals and  $y$  = the difference between Readings D or E and Reading C in the same tube.

$$\tan C = \frac{y}{x} = \frac{y_1}{5} \text{ or, } \frac{y_2}{10}$$

TABLE II. CALCULATION FROM DATA IN TABLE I\*

	TUBE 1 CONTROL	TUBE 2 KNOWN	TUBE 3 UNKNOWN
<i>Method 1</i>			
1.5 to 2 hours			
$y_1$	21	13	16
Tangent = $\frac{y_1}{5}$	4.2 = tan C	2.6 = tan K	3.2 = tan U
Angle from tan table	76.6° = C	69.0° = K	72.7° = U
C - K or U		7.6° = K'	3.9° = U'
Tangent from tan table		0.1334 = tan K'	0.0682 = tan U'
$\frac{\tan K'}{40} = m$		$\frac{0.1334}{40} = 0.003335$	
$\frac{\tan U'}{m} = \text{units} \times 1,000$			$\frac{0.0682}{0.003335} = 20.5$
1.5 to 2.5 hours			
$y_2$	43	25	32
Tangent = $\frac{y_2}{10}$	4.3	2.5	3.2
Angle from tan table	76.9°	68.2°	72.6°
Angle C - K or U		8.7°	4.3°
Tangent from tan table		0.1530	0.0752
$\frac{\tan K'}{40} = m$		$\frac{0.1530}{40} = 0.003825$	
$\frac{\tan U'}{m} = \text{units} \times 1,000$			$\frac{0.0752}{0.003825} = 19.6$
<i>Method 2</i>			
$y_4$		180	120
$f = \frac{y_4}{6.325\ddagger}$		$\frac{180}{6.325\ddagger} = 28.46$	
$\sqrt{z} = \frac{y}{f}$			$\frac{120}{28.46} = 4.216 = \sqrt{z}$
$z = \left(\frac{y}{f}\right)^2 = \text{units} \times 1,000$			$4.216^2 = 17.8$
<i>Summary</i>			
Theoretical units			0.0200
Method 1 with $y_1$			0.0205 = + 2.5%
Method 1 with $y_2$			0.0196 = - 2.0%
Method 2			0.0178 = -11.0%

\*In practice, since the position of the decimal point in the final result is evident by inspection, the decimal points may be ignored; however, it is necessary to carry the operations to four significant figures if the full accuracy of the method is to be attained.

†It is usually more convenient to express results in hundredths of a unit and divide by  $2 = \sqrt{4}$ , that is  $\frac{180}{2} = 90$ ,  $\frac{120}{90} = 1.333$ ,  $1.333^2 = 1.78$  hundredths of a unit = 0.0178 units per cubic centimeter.

when C is the angle which the control curve makes with the X axis,  $y_1$  is Reading D - Reading C for the control, and  $y_2$  is Reading E - Reading C. Determine the tangents of growth curves of each known (K) and unknown (U) from the appropriate readings on these tubes in precisely the same manner.

Look in a tangent table\* and find the values for the angles C, K, and U corresponding to tans C, K, or U.

Angle C - angle K = angle K' and angle C - angle U = angle U', the angles between curves of the control and each penicillin-containing culture.

From a tangent table obtain  $\tan K'$  which is directly proportional to the amount of penicillin. Therefore,  $\tan K'$  divided by the units of penicillin in the known expressed in thousandths is a constant, m.

In the example given, 0.04 unit penicillin was used in the known. The factor for calculation of the unknowns is found from:

$$\text{Concentration in thousandths of units} = m \tan K',$$

hence,

$$m = \frac{\tan K'}{40}$$

The concentration of penicillin in the unknowns can now be determined by substitution of  $\tan U'$  for each unknown for  $\tan K'$  in the previously mentioned equation.

Multiply by the dilution of the unknown for the value in the original material.

#### ALTERNATE METHOD OF CALCULATION WITHOUT USE OF TANGENT TABLES

From the trigonometric formula for the tangent of the difference of two angles it follows that:

$$\frac{\tan C - \tan K}{1 + \tan C \cdot \tan K} = \tan K'$$

and

$$\frac{\tan C - \tan U}{1 + \tan C \cdot \tan U} = \tan U'$$

The following is an example using data for  $\frac{y_2}{10}$  from Tables I and II for tans C, K, and U:

$$\frac{4.3 - 2.5}{1 + 4.3 \cdot 2.5} = \frac{1.8}{1 + 10.75} = \frac{1.8}{11.75} = 0.1532 = \tan K'$$

$$\frac{4.3 - 3.2}{1 + 4.3 \cdot 3.2} = \frac{1.1}{1 + 13.76} = \frac{1.1}{14.76} = 0.0745 = \tan U'$$

$$\frac{0.1532}{40} = 0.00383$$

$$\frac{0.0745}{0.00383} = 19.5 \text{ thousandths of a unit per cubic centimeter.}$$

\*Lange, N. A.: Handbook of Chemistry, ed. 6, Sandusky, Ohio, 1946, Handbook Publishers, p. 198 (appendix, mathematical tables, and formulas); or see other handbooks or trigonometry texts.

*Method 2.*—Subtract the corrected Reading F for the known penicillin solutions and for the unknowns from the corrected Reading F of the control, Tube 1. Call this difference  $y_4$ . Let  $z$  equal the concentration of penicillin in the colorimeter tube in hundredths of a unit per cubic centimeter. The difference in reading is directly proportional to the square root of the concentration. Therefore, Equation 1 expresses the relationship between  $y$  and  $z$ , when  $f$  is a factor, usually between 60 and 120, corresponding to the  $y_4$  value for 0.01 unit of penicillin per cubic centimeter and depending on the initial inoculum, the susceptibility of the organism, and the rate of growth.

$$\text{Equation 1: } y_4 = f \sqrt{\frac{2}{z}}$$

Determine the factor  $f$  by substituting the known concentration of penicillin ( $z$ ) expressed as hundredths of units and the corrected colorimeter difference reading,  $y_4$ , in the Equation 1 and solving for  $f$ . After  $f$  has been determined, substitute the  $y_4$  readings for each unknown and solve for the concentration  $z$ . A sample calculation is given in Table II.

*Modifications for Special Purposes.*—The technique previously described is suitable for the determination of penicillin in solution, in cerebrospinal fluid, in urine, and in *Penicillium notatum* cultures. Slight modifications are necessary for determination in blood serum, ascitic fluid, or pleural fluids and for greatest accuracy in urine and *P. notatum* cultures. The computation is the same for all, except that the dilution factor varies.

*Directions for Blood Serum:* Record the time of the last administration of penicillin. Separate the serum from a 5 to 10 c.c. blood sample collected in a rubber vial-capped centrifuge tube twenty to ninety minutes after the last dose of penicillin. Record the time of collection.

Into Tube 1, place 0.5 c.c. of sterile saline and 0.5 c.c. of the serum of the patient, taken just before dose of penicillin, or clear serum from a healthy person who has not been receiving neoarsphenamine or other trivalent organic arsenicals; into Tube 2, place 0.5 c.c. of standard penicillin solution and 0.5 c.c. of the same serum used in Tube 1; into Tubes 3, 4, 5, etc., place 0.5 c.c. of saline solution and 0.5 c.c. of each unknown serum. Add to each 4.0 c.c. of the broth culture.

If values below 0.1 unit per cubic centimeter are anticipated, it is better to use 1.0 to 2.0 c.c. of the serum and 3.5 or 2.5 c.c. of inoculated broth and to multiply by a dilution factor of 5.0 or 2.5 instead of 10 to get the units in the unknown serum.

*Directions for Cerebrospinal Fluid:* These are the same as for solutions of penicillin, except that larger quantities may be necessary if the concentration is low, and dilutions may be necessary if the concentration is high.

*Directions for Pleural or Ascitic Fluid:* Centrifugate and, if grossly purulent, pass the supernatant fluid through a Seitz filter to clarify and sterilize. Then proceed as for blood serum.

*Directions for P. notatum Cultures:* Make an accurate dilution of the centrifugated or Seitz-filtered\* medium, so that the estimated content in the final broth culture will be approximately 0.04 unit per cubic centimeter and will certainly lie between 0.01 and 0.1 unit per cubic centimeter. Use uninoculated equally diluted *P. notatum* medium for the control and known penicillin tubes and proceed as for penicillin solutions.

#### MODIFICATIONS OF TECHNIQUE

Certain simple adaptations of the standard technique previously described have been found useful and are herein listed. All procedures follow this standard technique except as noted. Results shown in Tables III to VI are consecutive and unselected and include normal routine manipulative errors. Dilutions were made with unselected tuberculin syringes.

TABLE III. RESULTS WITH TECHNIQUE FOR MAXIMUM SIMPLICITY\*

UNITS PER C.C.		PER CENT ERROR	UNITS PER C.C.		PER CENT ERROR
ADDED	FOUND		ADDED	FOUND	
0.080	0.073	- 9	0.020	0.016	-25.0
	0.064	-20		0.019	- 5.0
	0.081	+ 1		0.012	-40.0
	0.047	-41		0.014	-30.0
	0.049	-39		0.014	-30.0
	0.052	-35		0.010	-50.0
	0.096	+20		0.019	- 5.0
Av.	0.066	-17.5		0.025	+25.0
0.060	0.047	-21		0.019	- 5.0
	0.051	-15		0.018	-10.0
0.050	0.046	- 8		0.018	-10.0
				0.021	+ 5.0
0.040	0.040	0		0.027	+35.0
	0.041	+ 2.5		0.017	-15.0
	0.037	- 7.5		0.029	+45.0
	0.038	- 5.0		0.026	+30.0
	0.036	-10.0		0.023	+15.0
	0.039	- 2.5	0.023	+15.0	
	0.042	+ 5.0	0.015	-25.0	
	0.040	0.0	Av.	0.0192	- 4.0
	0.050	+25.0	0.010	0.005	-50.0
	0.040	0.0		0.005	-50.0
	0.042	+ 5.0		0.007	-30.0
	0.040	0.0		0.005	-50.0
	0.040	0.0		0.008	-20.0
	0.042	+ 5.0		0.009	-10.0
	0.042	+ 5.0		0.005	-20.0
	Av.	0.0406		+ 1.5	0.007
0.035	0.034	- 3.0		0.008	-20.0
				0.010	0.0
0.030	0.027	-10.0		0.006	-40.0
	0.027	-10.0		Av.	0.0068
0.025	0.022	-12.0			

\*All were computed from a single control and a single known containing 0.040 unit of penicillin per cubic centimeter. Accuracy is considerably increased if two knowns are included and the formula given under Technique for Maximum Range is used.

\*Centrifugation or filtration through a Seitz filter is necessary only when research accuracy is desired.

TABLE IV. RESULTS WITH TECHNIQUE FOR EARLIEST POSSIBLE RESULT\*

UNITS PER C.C.		PER CENT ERROR	UNITS PER C.C.		PER CENT ERROR
ADDED	FOUND		ADDED	FOUND	
0.500	0.220	-66.0	0.030	0.33	+10.0
0.100	0.094	- 6.0	0.020	0.022	+10.0
	0.054	-46.0		0.030	+50.0
0.080	0.078	- 2.7		0.022	+10.0
	0.075	- 3.1		0.021	+ 5.0
	0.094	+17.5		0.026	+30.0
	0.054	-32.7		0.021	+ 5.0
0.060	0.061	+ 1.7		0.023	+15.0
	0.040	-33.3		0.030	+50.0
0.040	0.040	0.0		0.019	- 5.0
	0.040	0.0		0.023	+15.0
	0.040	0.0		0.021	+ 5.0
	0.040	0.0		0.018	-20.0
	0.031	-22.5		Av.	0.023
	0.051	+22.5	0.040†	0.036	-10.0
	0.040	0.0		0.036	-10.0
	0.040	0.0		0.027	-32.5
	0.023	-42.5		0.036	-10.0
	0.040	0.0		0.039	- 2.5
	0.031	-22.5		0.031	-22.5
	0.040	0.0		0.043	+ 7.5
	0.040	0.0		0.039	- 2.5
	0.040	0.0	0.035	-12.5	
Av.	0.0394	- 1.5	Av.	0.0358	-12.5

\*All results were available within two hours.

†These were computed from 0.020 unit per cubic centimeter known, all others from a 0.040.

TABLE V. RESULTS WITH ROUTINE TECHNIQUE

UNITS PER C.C.		PER CENT ERROR	UNITS PER C.C.		PER CENT ERROR
ADDED	FOUND		ADDED	FOUND	
0.080	0.076	- 5.0	0.0200	0.0198	- 1.0
	0.101	+25.1		0.0186	- 7.0
	0.093	+13.2		0.0189	- 5.5
	0.138	+72.5		0.0189	- 5.5
	0.054	-32.5		0.0216	+ 8.0
Av.	0.0924	+15.5		0.0275	+37.5
0.060	0.066	+10.0		0.0192	- 4.0
	0.047	-21.7		0.0206	+ 3.0
0.040	0.040	-20.0		0.0207	+ 3.5
	0.045	+12.5		0.0210	+ 5.0
	0.037	- 7.5		0.0241	+ 2.5
	0.040	0.0		0.0174	-13.0
	0.044	+10.0		0.0243	+21.5
	0.032	-20.0		0.0186	- 7.0
	0.036	-10.0		0.0195	- 2.5
	0.040	0.0		0.0232	+16.0
	0.040	0.0		0.0185	- 7.5
	0.036	-10.0		0.0186	- 7.0
	0.036	-10.0		0.0196	- 2.0
	0.040	0.0	Av.	0.02056	+ 2.8
	0.040	-10.0	0.0100	0.0110	+10.0
	0.040	0.0		0.0130	+30.0
Av.	0.0387	- 3.25		0.0076	-24.0
0.035	0.034	- 3.0		0.0112	+12.0
0.030	0.028	-6.7		0.0103	+ 3.0
	0.026	-13.3		0.0090	-10.0
	0.024	-20.0		0.0079	-21.0
0.025	0.024	-4.0		0.0080	-20.0
	0.022	-12.0		0.0100	0.0
			Av.	0.0070	-30.0
				0.0095	- 5.0

*For Maximum Simplicity.*—Carry out all incubations in an incubator. Omit all readings except Reading F at 4 to 30 hours, preferably 18 to 24 hours, after start of incubation. Compute by Method 2 only, using nomograms.<sup>1</sup> Accuracy for a single reading is about  $\pm 25$  per cent, as shown by examples in Table III.

*For Earliest Possible Result.*—After 1-hour incubation read, at 15-minute intervals, Readings C, D, and E. Calculate by Method 1 on basis of first half-hour period in which the successive 15-minute readings on the same tube agree within two points and differ from control by more than two points. Results are given in Table IV. Note that accuracy is much less at low concentrations, but the readings may be continued to follow the standard technique.

*For Routine Technique.*—Take Readings C and E at the beginning and end of any 1-hour period, beginning between 1.5 and 3.5 hours after the tubes are placed in the water bath. Results are shown in Table V. Accuracy of a single determination is about  $\pm 10$  per cent.

To simplify laboratory procedure, inoculated broth may be introduced into the culture tubes by a pipetting machine and micropipettes or drops from calibrated needles utilized for introduction of concentrated samples.

TABLE VI. RESULTS AT EXTREMES BY TECHNIQUE FOR MAXIMUM RANGE\*

METHOD 1		METHOD 2		AVERAGE	PER CENT ERROR
UNITS PER C.C.		UNITS PER C.C.			
ADDED	FOUND	FOUND			
0.256	0.245	0.145	0.195	- 20.0	
0.128	0.125	0.141	0.133	+ 2.0	
0.100	0.080	0.078	0.079	- 21.0	
	0.087	0.084	0.086	- 14.0	
0.080	0.077	0.082	0.079	- 1.2	
	0.081	0.079	0.080	0.0	
	0.092	0.092	0.092	+ 15.0	
0.064	0.064	0.077	0.070	+ 9.4	
0.060	0.062	0.061	0.061	+ 1.7	
	0.057	0.064	0.061	+ 1.7	
0.008	0.0081	0.0082	0.0082	+ 2.5	
	0.0094	0.0148	0.0121	+ 51.0	
	0.0063	0.0097	0.0080	0.0	
0.004	0.0061	0.0050	0.0055	+ 27.5	
	0.0020	0.0013	0.0017	- 57.5	
	0.0037	0.0052	0.0045	+ 12.5	
0.002	0.0019	0.0014	0.0017	- 15.0	
	0.0023	0.0035	0.0029	+ 45.0	
	0.0011	0.0011	0.0011	- 45.0	
0.001	0.0011	0.0017	0.0015	+ 50.0	
	0.0030	0.0017	0.0023	+115.0	
0.0005	0.0006	0.0004	0.0005	0.0	

\*Accuracy in middle range is intermediate between routine technique and technique for maximum accuracy.

*For Maximum Sensitivity to Low Concentrations.*—Use Oxford *Staph. aureus* strain H. Insure that inoculum gives  $B' - A$  reading of 10 to 12 or less and that the known contains a final concentration of 0.008 unit per cubic centimeter of standard penicillin. Results are shown in Table VI.

*For Maximum Accuracy.*—Determine the approximate concentration. Dilute, with precision-calibrated glassware, all unknowns to a calculated final concentration of 0.04 unit per cubic centimeter. Run control, known, and each unknown in quadruplicate, using the standard or routine technique and average results.

Our results by this method agree within  $\pm 5$  per cent. Duplicate determinations on standard penicillin agreed within  $\pm 2.5$  per cent with one control, one known, and three replicate unknowns. Inspection of the 0.04 unit per cubic centimeter block of Table V will indicate the results available by any number of replicate determinations in this concentration range.

*For Maximum Range.*—Use a series of knowns ranging in final concentration by 0.02 unit steps from 0.2 to 0.02 unit per cubic centimeter and by 0.002 unit steps from 0.01 to 0.002 unit per cubic centimeter. Use as knowns the nearest higher and lower concentrations, compute by both Methods 1 and 2, and average the results.

Our experience has shown that the concentration by Method 2 is more accurately given by the relation

$$y_4 = f \sqrt{z} - b$$

where  $b$  is an arbitrary constant. To utilize this formula, two knowns are needed. The following example illustrates the method of determination of constants:

For 0.040 unit per cubic centimeter  
For 0.010 unit per cubic centimeter

$$\begin{aligned} y_4 &= 192 \\ y_4 &= 84 \end{aligned}$$

Expressing concentration  $z$  in hundredths of units and substituting these values,

$$(1) \quad 192 = f\sqrt{4} - b$$

$$(2) \quad 84 = f\sqrt{1} - b$$

---

Subtracting (2) from (1)  $108 = f$ .  
Substituting  $f = 108$  in equation (1) or (2).

$$b = 24$$

Hence, in this example,  $y_4 = 108 \sqrt{z} - 24$ . Results routinely obtained by this method of computation are shown in Table VI.

#### TECHNIQUE FOR STREPTOMYCIN ASSAY

The method is equally satisfactory for streptomycin assay. The only modifications necessary are the use of two knowns containing streptomycin and making Reading F for Method 2 at 4 to 6 hours instead of the next day.

Knowns are prepared with a final concentration of 1 unit ( $1\mu\text{g}$ ) and 3 units ( $3\mu\text{g}$ ) per cubic centimeter. Calculation by Method 1 is identical with that described for penicillin.



If Method 2 is used, the concentration is *directly* proportional to the  $y_4$  difference in colorimeter reading; hence,

$$y_4 = f z$$

when  $z$  is the streptomycin concentration in units per cubic centimeter. If it be desired to read the streptomycin cultures the next day, a known of 10 units per cubic centimeter must be used. Nomograms<sup>1</sup> may be used for calculation.

The effective range for streptomycin is 0.2 to 20 units per cubic centimeter in the final culture. Sensitivity may be increased for low concentrations by the use of up to 50 per cent serum and correspondingly less broth culture medium.

#### TECHNIQUE FOR TRIVALENT ORGANIC ARSENICALS

The trivalent organic arsenicals used in the treatment of syphilis have been shown<sup>3</sup> to be very effective antibacterial agents against all strains of staphylococci investigated, as well as against hemolytic streptococci, some strains of *Hemophilus influenzae*, and many strains of *Streptococcus viridans*. Therefore, the method herein described may be used for the assay of any of these trivalent organic arsenicals. The technique for Method 1 is identical with that for penicillin or streptomycin and for Method 2 it is the same as that described for streptomycin, since the  $y_4$  differences are directly proportional to the concentrations. The ideal concentration of the known for both methods with neoarsphenamine is 1.5  $\mu\text{g}$  per cubic centimeter in the final culture. The ideal concentration for mapharsen is 0.75  $\mu\text{g}$  per cubic centimeter. For the other trivalent organic arsenicals, the activity against bacteria is proportional to the arsenic content relative to that of neoarsphenamine,<sup>3</sup> so that knowns for these should be so diluted that the final culture contains 0.3  $\mu\text{g}$  per cubic centimeter of arsenic. The effective range for neoarsphenamine is from 0.2 to 3.0  $\mu\text{g}$  per cubic centimeter; therefore, unknowns should be diluted to an estimated concentration of 2 to 30  $\mu\text{g}$  per cubic centimeter when 0.5 cubic centimeter is used with 4.5 cubic centimeter of broth culture.

#### DETERMINATION OF SERUM LEVELS OF ANTIBACTERIAL AGENTS DURING THE ENTIRE TIME PERIOD BETWEEN DOSES

Except when antibacterial agents are given by continuous drip, a single serum level does not indicate the concentration at any time except the moment when the blood was taken. However, the levels at other times may be roughly computed by the factors given elsewhere.<sup>1</sup> They can be accurately determined by making two determinations an hour or two apart, the first one 30 minutes to an hour after administration of the drug. Using the equal interval scale for time and the logarithmic scale for concentration, plot on polycyclic semilog graph paper these two points and draw a line through them. For the trivalent organic arsenicals, follow the same procedure, plotting the results on log log paper. The serum levels at any time after the initial mixing may now be read directly from the graph.

## TECHNIQUE FOR QUANTITATIVE BACTERIOLOGIC STUDIES

It seems possible that this technique could be used equally well for quantitative studies of the effects of any variable on bacterial growth, and the results would be available quickly instead of having to wait for colony counts and with far greater accuracy than colony counting technique affords. Growth could be stopped at any desired stage and quantitative studies made on the medium.

## THE EFFECTS OF VARYING THE INOCULUM

Any clear, fluid, bacteriologic medium may be used if light transmission is not too low. Hartley broth gives even more satisfactory results, and because it does not contain dextrose might be preferable in testing *P. notatum* cultures which may contain notatin. The bactotryptose medium was selected because it was readily available and easily prepared, is more or less standard in composition, is already buffered, and gives very rapid growth to high turbidity. Double- or triple-strength bactotryptose broth gave no better results than the standard-strength broth. Addition of serum does give better results than the standard-strength broth. One per cent and 2 per cent bactopectone broth gave slower growth to lower turbidities. The faster the growth, the more uniform its rate, and the higher the final turbidity, the better are the results with penicillin; and the slower the growth, the greater is the sensitivity to streptomycin. For penicillin  $y_3$  values of 30 to 70 are satisfactory, and for streptomycin  $y_2$  values of 20 to 50 are satisfactory. Still better media no doubt can be discovered.

## THE EFFECT OF VARYING THE INOCULUM

These effects are shown graphically in Fig. 2 for penicillin. The lower the inoculum, the slower is the start of the straight-line phase of growth, the more rapid is the final growth rate attained, the more sensitive is the technique for low concentrations, the more accurately do the penicillin effects correspond to the equations, and the greater are the  $y_4$  and tangent differences per unit concentration of penicillin. However, with low inocula, a slight difference in the size of the inoculum or the blank reading of the colorimeter tube introduces a much greater error in the result, and the range for high concentrations of penicillin is reduced by early cessation of growth. Inocula corresponding to a  $B' - A$  reading of 30 or over are unsatisfactory because of small differences in growth rate with large differences in penicillin concentration. For most purposes an inoculum corresponding to a  $B' - A$  reading of 12 to 15 is most satisfactory.

## THE EFFECTS OF VARYING THE STRAIN OR SPECIES OF ORGANISM USED

Much more work needs to be done on this. The ideal organism for routine use should be only moderately sensitive to penicillin action, should grow rapidly to high turbidities, should show no tendency to produce variants, and should remain in the straight-line phase of growth for a long time on simple and inexpensive media. The Oregon-J strain of hemolytic *Staph. aureus* more nearly fulfills these requirements than any other organism so far tested and is very satisfactory also for streptomycin and trivalent organic arsenicals. The strain of

beta hemolytic streptococcus investigated did not grow to high enough turbidities or rapidly enough; the Oxford *Staph. aureus* strain II, while slightly superior for very low concentrations of penicillin, did not stay in the straight-line phase of growth long enough and ceased growth at a turbidity reading of 200 versus 250 for the Oregon-J strain. The National Institute of Health's strain 209 P had the same defects as the Oxford strain H; however, it was not as sensitive to low concentrations, although it was more sensitive than the Oregon-J strain. Other strains of staphylococci, strains of *Bacillus subtilis*, and pneumococci have been investigated and were found unsuitable.

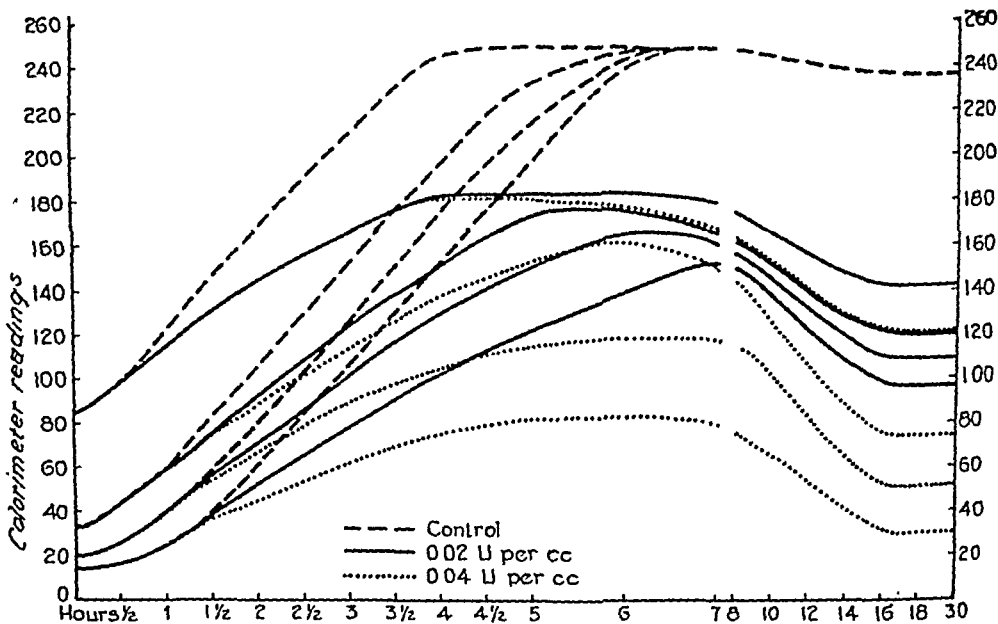


Fig. 2.—Effects of variations in size of inoculation on growth curves of the control and penicillin-containing cultures.

#### THE EFFECTS OF VARYING THE TIME OF READINGS

As may be seen from Fig. 2, the longer the interval between readings used in the computation by Method 1, the smaller the relative error in the photoelectric colorimeter readings, but the greater the error due to departure of the growth curve from a true straight-line curve, especially with high concentrations. The 1-hour period is best for the entire range, but a half-hour period is best for concentrations above 0.06 unit per cubic centimeter with the Oregon-J strain. Too early initial readings introduce error by including a part of the lag phase of penicillin action or of the prestraight-line growth curve, and too late readings introduce error by including nonstraight-line portions of the growth curves. With the standard technique and the Oregon-J strain, growth is essentially straight line from  $1\frac{1}{4}$  to 4 hours with concentrations below 0.06 unit per cubic centimeter and departs least from a straight-line curve between 1.5 and 3.5 hours.

In calculation by Method 2,  $y_4$  differences increase in significance and reliability rapidly from 3 hours, when they first become significant for high concentrations, to 6 hours and then slowly until 17 hours, after which they change very little in the next 24 hours.

#### THE EFFECTS OF VARYING THE TIME SCALE

The reason for choosing 6-minute units is that it brings the tangents into a convenient part of the tangent table and involves dividing by 10, which is merely pointing off a decimal place. It is not important that these be exact 6-minute units; equally accurate results are obtained if the time interval is somewhat shorter or longer than an hour and 10 is still used as the divisor, provided that the time interval between readings is the same for each tube.

#### THE EFFECTS OF VARYING THE FILTER

Any filter may be used, but the advantages of the No. 66 filter are that it transmits red and yellow light very well, giving a low blank that is little affected by the yellow colors of the medium, penicillin solutions, blood serum, urine, or the red colors of slightly hemolyzed serum. For certain research purposes, where very low concentrations are to be determined, a green No. 54 filter is preferable, since it gives a higher difference for small changes in turbidity; however, the blanks will be higher with this filter. Still greater accuracy was obtained with the blue No. 42 filter and Hartley broth, but the bactotryptose broth gives too high a blank A reading.

#### USE OF OTHER PHOTOELECTRIC COLORIMETERS

The principle of the method is applicable directly to any photoelectric colorimeter having a logarithmically spaced scale and can be adapted to other types

by the formula\*  $(2.000 - \log_{10} T) \times 500 \times \frac{12}{\text{inside tube diameter in mm.}} =$

reading on Klett-Summerson instrument. However, the Klett-Summerson instrument is strongly recommended because of the convenient size of the tubes, the large number of scale divisions, the great rapidity with which readings may be made, and the high accuracy without sacrifice of range.

#### COMPARISON WITH OTHER METHODS

*In comparing the results in the tables with those of other methods, it is important to remember that these† are results on a single unknown, when many unknowns were determined from a single control and a single known; whereas the results reported for most methods are the averages of several replicates or dilutions of an unknown compared with a series of controls and knowns.*

\*When  $(2.000 - \log_{10} T)$  is the formula for deriving optical density from per cent transmission (T), and 12 mm. is the inside diameter of the Klett-Summerson tube. The Klett-Summerson scale is calibrated in divisions  $500 \times$  optical density.

†Except results in Table VI which are based on two knowns.

## SUMMARY

A quantitative method of penicillin assay is described which is flexible enough to compare favorably with any published method in simplicity, sensitivity, accuracy, range, specificity, and speed with which results are available.

It is based on turbidimetric determination, using a new principle of computation.

It is adapted to all types of penicillin assay now made and to quantitative assay of other potent bacteriostatic or bactericidal agents. Techniques for streptomycin and trivalent arsenicals are given.

The method has the advantage of employing materials and supplies already available in most laboratories and techniques with which the average laboratory technician is familiar. Results of plus or minus 25 per cent accuracy may be available within 2 hours and of plus or minus 10 per cent accuracy within 2.5 to 3 hours. For most applications there is no interference by penicillin-resistant contaminants. It will detect as little as 0.001 unit per cubic centimeter of penicillin with plus or minus 100 per cent accuracy. When performed for maximum accuracy, error can be reduced below plus or minus 5 per cent. It will cover a fourfold range with plus or minus 10 per cent accuracy, a sixfold range with plus or minus 25 per cent accuracy, and a hundredfold range with plus or minus 100 per cent accuracy at the extremes.

## REFERENCES

1. Osgood, E. E., and Graham, S. M.: A Simple Rapid Method for Assay of Bactericidal and Bacteriostatic Agents: With Techniques for Assay of Penicillin, Streptomycin, Neocarsphenamine, Mapharsen and Other Trivalent Organic Arsenicals, *Am. J. Clin. Path.* 17: 93-107, 1947.
2. Foster, J. W., and Woodruff, H. B.: Microbiological Assay of Penicillin. I. Methods of Assay, *J. Bact.* 46: 187-202, 1943.
3. Osgood, E. E.: The Comparative Effectiveness of Arsenical Compounds and Sulfonamide Drugs Against Bacterial Infection, *J. LAB. & CLIN. MED.* 28: 953-962, 1943.
- Osgood, E. E.: What the General Practitioner Should Know About the Chemotherapy of Bacterial Infection, *Canad. M. A. J.* 50: 1-8, 1944.

# AN EXPERIMENTAL METHOD FOR OBTAINING AN ULTRAFILTRATE OF THE BLOOD

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ULTRAFILTRATION may be a useful approach to the investigation of simultaneous clearances with the kidney and in the study of bound and unbound blood constituents. Ultrafiltration permits extraction of a true ultrafiltrate<sup>1</sup> and can be considered as analogous in principle to glomerular filtration. Geiger<sup>2</sup> has described an ingenious device consisting of two layers of collodion supported by two perforated metal sheets that he connected to the carotid artery of a dog. The blood was circulated between the collodion layers and an ultrafiltrate extracted. It is possible that an improved ultrafilter may have some merit, when properly developed, as a supplement to the excretory function of the kidney.

Our purpose was to develop an ultrafilter which could duplicate glomerular function in a dog, both qualitatively and quantitatively, to be used in an attempt to prolong the life of uremic animals. We decided to use cellophane tubing as the filter membrane, as it appears to have advantages over other materials used in the past. Recent reports have shown that it has a very reproducible and consistent porosity, that sterilization does not greatly alter its permeability, that it has a neutral reaction, and that it can withstand high pressures when properly supported.<sup>3-8</sup> From a practical standpoint cellophane tubing is inexpensive, is available in several thicknesses and diameters, and is relatively easy to handle.

We have constructed an apparatus which permits the flow of blood from an animal through cellophane tubes arranged to provide a maximal filtering surface per unit volume of blood. To increase the rate of water and crystalloid extraction, the cellophane tubes are submitted to an effective filtering pressure of approximately 800 mm. of mercury. This equipment has been successful in collecting the ultrafiltrate of circulating blood in quantities up to 1,200 c.c. per hour.

## DESCRIPTION OF THE APPARATUS

The apparatus consists of a filter, a vacuum chamber, and a pump to circulate the blood. These are illustrated in Figs. 1 to 8 and details are given in the legends.

*The Filter* (Figs. 1 to 4).—The filter is made up of twenty identical filter tubes connected in parallel. Each filter tube consists essentially of a flat cello-

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phane tube\* passed between two strips of nylon tape† which are sewed together down their full length. This is then sandwiched between two stainless steel strips which are held together by a slotted clamping bar on each side. Filtering pressure is obtained by placing the completely assembled filter tube in a vacuum chamber, bringing the blood inlet and outlet tubes out through a seal, rather than by subjecting the blood to an increased positive pressure.

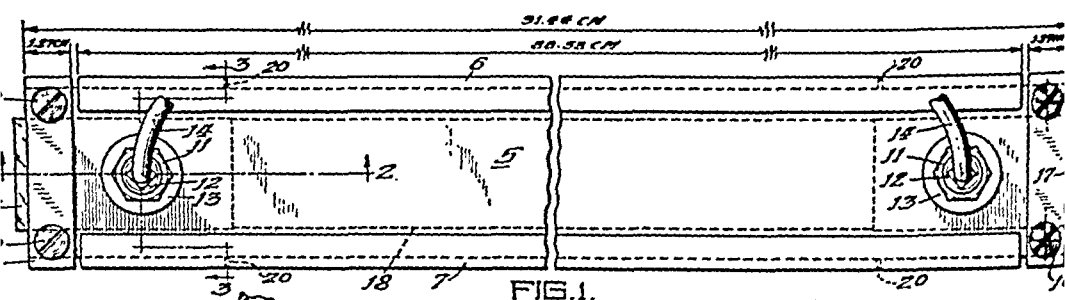


FIG. 1.

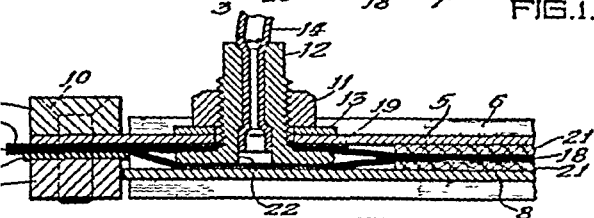


FIG. 2.

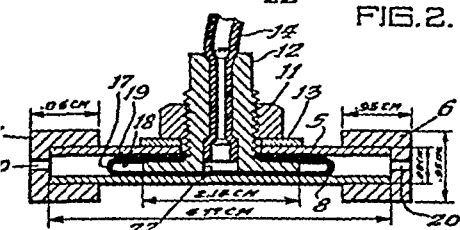


FIG. 3.

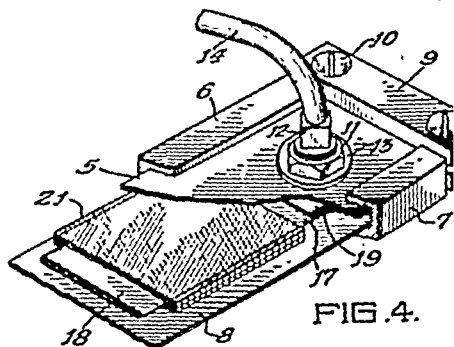


FIG. 4.

Figs. 1 to 4.—Details of the filter: A cellophane tube (18) 21.4 mm. in diameter and 0.02 mm. in thickness is passed between two strips of nylon tape (21), which are sewed down their full length on both sides so as to form a continuous tube over the cellophane. A short piece of cellophane tubing (17) is passed over each end of the previous one. Two flanged stainless steel connections (12),\* with their rubber tubes (14), are passed through holes previously pierced in the cellophane tube. A thin rubber gasket (19) is placed over the flange of each connection, and the latter are then screwed to a stainless steel strip (5) with a washer (13) and a nut (11). This strip has two holes in it, 11 mm. in diameter and 83.9 cm. apart, for the purpose. Each end of the cellophane tube is closed with two stainless steel bars (9 and 15) with a rubber gasket (16) between them. These bars are fastened with two screws (10). Another strip (8), of the same width as (5) but 2.8 cm. shorter, is placed under (5) and both are inserted into the slots milled in the clamping bars (6 and 7). Holes (20), 1 mm. in diameter, at intervals of 9.5 cm. permit the drainage of the filtrate.

\*Between the flange of the connection (12) and the cellophane tube (18) there is a space, not shown in Figs. 2 or 3, through which the blood finds its way when it circulates within the filter.

The principle of the filter is simple. The cellophane tube through which the blood circulates acts as a semipermeable membrane. It is separated from the steel strips by the nylon tape which, by virtue of its weave, forms a porous chamber through which the vacuum can act to reduce the pressure on the exterior of the cellophane tube.

\*Manufactured by Visking Co., Chicago, Ill.

†Kindly supplied by Phoenix Trimming Co., Chicago, Ill.

The capacity of each filter tube is 30 c.c., but this can be reduced by placing filter paper strips of appropriate thickness between the nylon tape and the steel strips. The filtration surface of each unit is 400 sq. cm., approximately. Under a filtering pressure of 800 mm. Hg and with a blood flow between 15 and 100 c.c. per minute, about 60 c.c. of ultrafiltrate per hour are obtained. If more ultrafiltrate is desired, several filters like the one described may be used in parallel (Fig. 5) connected by a collector tube. If the filter is to be used without

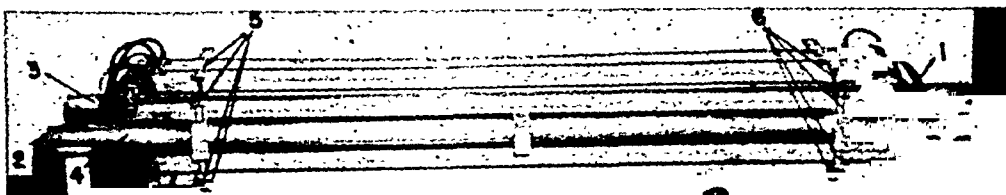


Fig. 5.—Twenty filter units connected in parallel with the collector tubes (1), by which blood is distributed to and collected from them. The tube (2) serves as an inlet or outlet for the blood in the whole filter. The filter tubes are clamped to an aluminum frame (3 and 4) with the metal strips and screws (5 and 6).

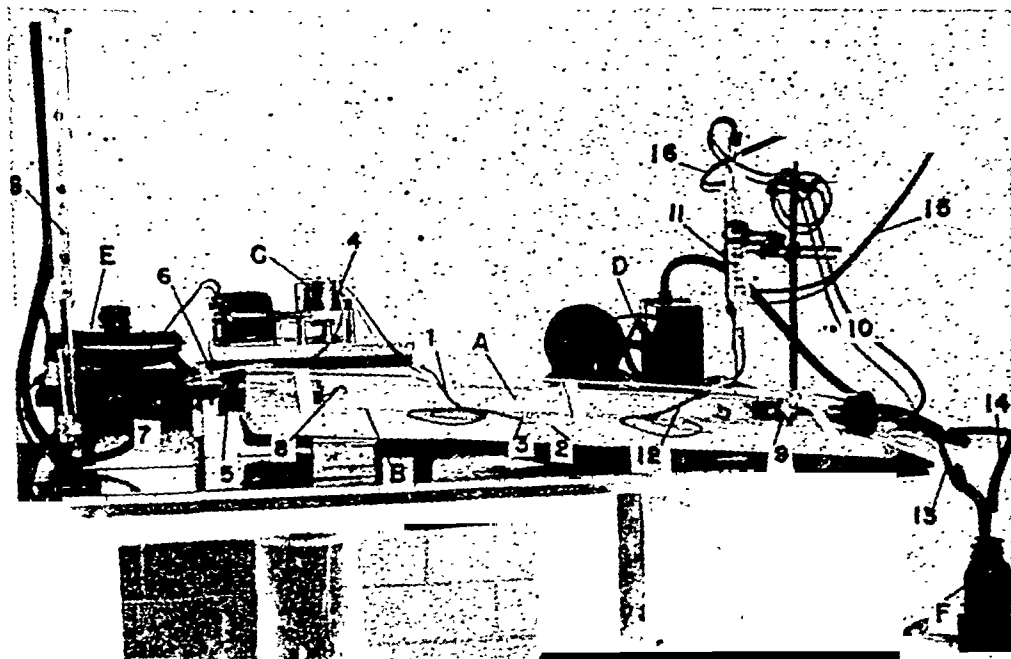


Fig. 6.—The apparatus assembled and ready for use. A, A single filter unit; B, the vacuum chamber; C, the blood pump; D, the vacuum pump; E, the rheostat; F, the bottle for collecting the ultrafiltrate; G, a mercury manometer connected to the vacuum chamber. The inlet tube (1) is connected through a wide cannula (2) and a three-way stopcock (3) to the femoral artery of the dog; (4) is the special rubber tubing used in the blood pump; (5) is the stopper with two tubes; (6) is the tube through which blood is pumped into the filter; (7) is the tube connected with the mercury manometer; (8) is the inlet tube to the filter; (9) is the outlet tube from the filter; (10) is the outlet tube from the chamber, leading to (11) an ordinary condenser used to warm the blood before being returned to the animal through tube (12). This latter tube also has a three-way stopcock and a cannula. This cannula is connected with the femoral vein of the animal. The bottle (F) receives the ultrafiltrate from the chamber through a rubber tube (13) and is also connected to the vacuum chamber by another tube (14). Other tubes (15 and 16) carry warm water to and from the condenser.



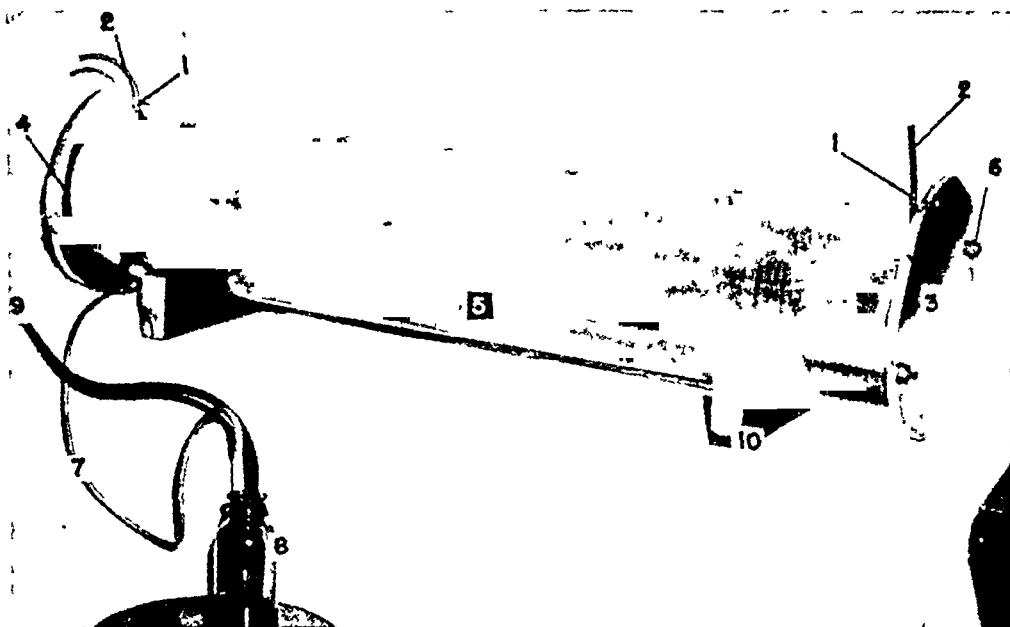


Fig. 7.—The large vacuum chamber employs a brass cylinder, 122 cm. in length, 25.3 cm. in inside diameter, and 3 mm in thickness. The metal tubes (1) of 8.5 mm. bore are slightly smaller than the rubber tubes (2) (Fig. 5, 2), in order to provide a perfect seal when the chamber is evacuated. The aluminum plates (5) seal the ends of this chamber with the rubber gasket (4) and are tightly held in place with the rods (3) and nuts (6). A tube (7) drains the ultrafiltrate into the collector bottle (8). Another tube (9) connects with the vacuum pump (not shown). The two supports (10) differ in height to permit the ultrafiltrate to drain toward the filtrate collecting tube (7).

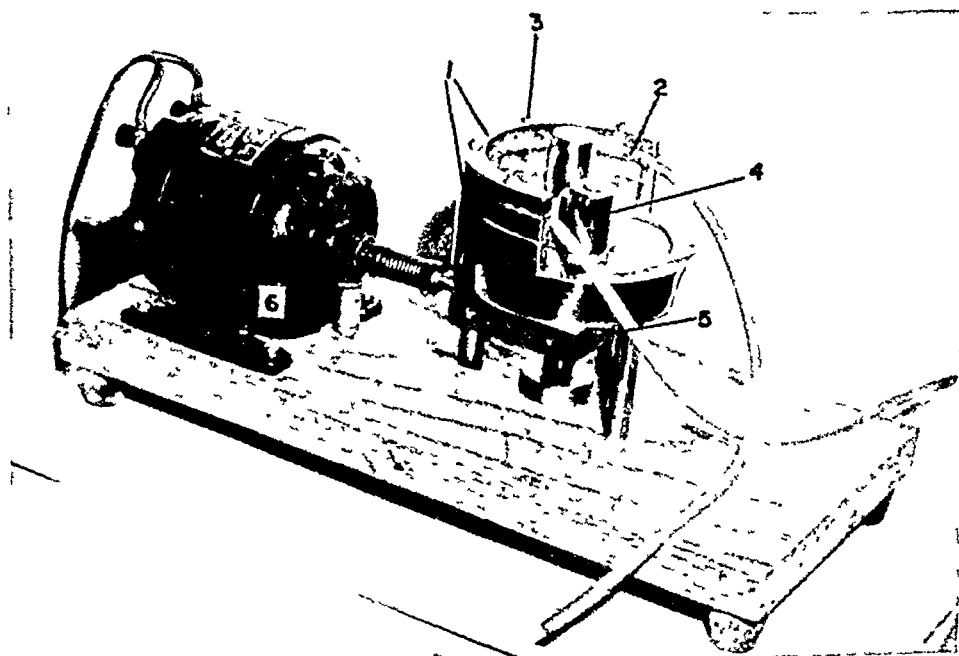


Fig. 8.—The pump consists of two semicircles of plated iron (1) between which the special tube (2), a rubber tube with a side piece of rubber that is held by the iron semicircles, is fastened with four screws (3). Rollers (4) milk the tube causing the blood to circulate. Gears (5) connect the axis of the roller arm with the motor (6).

circulation of blood or when a small amount of blood is to be ultrafiltered, the outlet tube is clamped after the filter has been filled.

*The Vacuum Chambers* (Figs. 6 and 7).—Two vacuum chambers were used. For use with one or two filter units a long glass tube was employed, which was sealed by a rubber stopper at each end. A glass cannula through each stopper provides a connection for the inlet and outlet tubes of the filter unit, and two more cannulae supply a means of exhausting the tubes and of collecting the ultrafiltrate.

The large chamber, for use with up to twenty filter tubes, is a large diameter brass tube closed at each end by an aluminum disk with a rubber gasket. A seal for the rubber inlet and outlet tubes of the filter is formed by drawing each through a short metal tube which is soldered into the body of the brass tube. The inside diameter of these tubes was made slightly smaller than the outside diameter of the rubber tubing to insure a good seal. Another tube soldered into the brass tube connects with a collector bottle for the ultrafiltrate.

*The Blood Pump* (Fig. 8).—This pump, similar to the one described by DeBaker,<sup>3</sup> consists essentially of two semicircles of plated iron, one on top of the other, between which a special rubber tube\* is clamped. Two rollers, driven by an electric motor, through a worm gear, milk approximately 2 c.c. of blood through this tube in each revolution. Among the advantages of this type of pump are that a nonpulsating flow is obtained and that there is little trauma to the blood cells.

#### TECHNIQUE IN ASSEMBLING THE EQUIPMENT†

The technique in assembling the equipment must be followed rigorously for successful results. A length of cellophane tubing is cut from the roll and is passed between the strips of nylon tape, leaving an extra 15 cm. of cellophane on each side. Two short pieces of cellophane tubing are opened‡ and placed over the ends of the longer tube to re-enforce it at these points. A dusting of starch powder is used to permit the short pieces to slide easily. The long tube is opened and a hole is punched near each end and on the same side of the cellophane tube with a special punch, 11 mm. in diameter, the same distance (83.9 cm.) apart as the holes in the strip (5). In order to make these holes, an ordinary wooden tongue depressor is introduced into the long tube and the punch is pressed against it through the double cellophane. The stainless steel connections (12) with their rubber tubes are passed through these holes, a thin rubber gasket (19) is placed above the cellophane, and the connections (12) are screwed tightly to the strip (5); then both ends are closed by tightly screwing the two bars (15 and 9) together with a rubber gasket (16) in between. The strip (8) is placed below and both strips are introduced into the slots of the clamping bars (6 and 7).

\*Obtained from A. S. Aloe Co., St. Louis, Mo.

†The numbers refer to Figs. 1 to 4.

‡Dry cellophane tubing is opened by applying a small strip of adhesive or transparent tape to each side of the flat cellophane tube about 1 cm. from the end, allowing the tape to project a little beyond the tube. The strips of tape are then pulled gently apart, thus separating the cellophane layers at the end of the tube. Blowing into the tube at this point will then open the tube down its entire length.

The unit so assembled is soaked in cool tap water for half an hour and then is checked for leaks by connecting one of the rubber tubes (14) with a source of air under approximately  $1\frac{1}{2}$  atmospheres of positive pressure. The patency of the filter is checked first by watching the escape of air from the second rubber tube. If the filter is not patent, the air is shut off and turned on again, increasing the pressure slowly. If this does not open the filter, injection of saline with a syringe may accomplish the purpose.

Leaks in the system are manifested by small bubbles coming out while air is injected under pressure with the outlet tube clamped off. These must be differentiated from trapped air in the system. Trapped air disappears after a time but air from a leak continues to escape. There are three sources of leaks:

1. Holes in the cellophane, which were found in one out of ten tubes of cellophane employed by us. Such leaky tubes must be replaced.

2. Inadequate tightening of the connections (12) or of the end clamps (15). A continuous stream of air bubbles locates the loose connection, and these leaks usually disappear after retightening.

3. Wrinkles in the cellophane at the ends (17). Under these conditions air continues to escape despite retightening. In such cases removing the end pieces (15) and smoothing out the cellophane generally corrects the leakage.

The connections (12), all rubber tubing, glass cannulae, and adaptors used in the experiment, were rendered pyrogen-free by boiling for thirty minutes in 4 per cent NaOH, rinsing with tap water, boiling for fifteen minutes in 0.5 per cent acetic acid, and finally rinsing again with tap water.

#### CHARACTERISTICS OF THE FILTER TUBE

In order to determine its characteristics, a filter tube was placed in the glass vacuum chamber and the inlet and outlet tubes each were connected with a graduated bottle containing citrated human blood. Altering the relative height of the two bottles changed the pressure and the rate and direction of blood flow. Further pressure and flow changes were obtained by using a screw clamp on the outlet tube.

Filtration under the same conditions was performed with two filter tubes which were identical except that the capacity of one was 15 c.c., the other 30 c.c. The filtration rates of both were the same. Nevertheless, when a large number of units are used in parallel, the total amount of ultrafiltrate is sometimes less than would be obtained if the same number of units were functioning separately. The probable reason for this is that, as the resistance of a large number of filter tubes in parallel is very low, with a relatively small blood flow (up to 150 c.c. per minute for 20 units) some of the tubes are not supplied with circulating blood and so do not function.

No change in the filtration rate was observed: (1) as a result of sterilization\* with steam at 250° F. for fifteen minutes; (2) with continued filtration

\*Sterilization was performed by connecting the inlet of the filter with a pressure cooker after the desired temperature and pressure was attained and clamping the outlet of the filter.

over ten hours; (3) with blood flow varying from between 15 and 100 c.c. per minute; and (4) with ultrafiltering liquids of different viscosity (namely, blood, and saline solution).

The blood used in filtration was examined microscopically on a number of occasions after centrifuging, and no hemolysis was observed.

Using a vacuum of 35 mm. Hg and varying the positive pressure applied to the circulating blood, the following results were obtained in the same filter:

EFFECTIVE PRESSURE IN MM. HG	FILTRATION RATE IN C.C. PER HOUR
760	54
760	56
780	60
805	60
825	60
825	59
875	63
925	70

The filtration rate was 5 c.c. per hour in another experiment in which only 75 mm. Hg of positive pressure was applied and no negative pressure was used.

As expected, these observations show that there is a proportionality between the pressure and the amount of ultrafiltrate formed.

#### NATURE OF THE ULTRAFILTRATE

The ultrafiltrate obtained from human blood and that of dog blood were analyzed in several instances for urea, nonprotein nitrogen, and proteins. It was found to be protein-free and with the N.P.N. and urea in equilibrium with the blood. In later experiments employing dogs certain other ultrafiltrate constituents were analyzed and the results are shown in Table I.\*

#### ACTION OF THE ULTRAFILTER WHEN CONNECTED TO A DOG

*Method.*—Two types of experiments were performed. In the first type, a single ultrafilter tube was connected with a normal dog. In the second, the large unit with 20 such units in parallel were connected with a nephrectomized, uremic dog. For the latter type of experiment the dogs were bilaterally nephrectomized three days previously; they were injected daily with 100 c.c. of 50 per cent solution of glucose intravenously and with 100 c.c. of 5 per cent glucose, 50 c.c. of saline, and 50,000 units of penicillin subcutaneously. Blood volume determinations were made at the beginning and at the end of the experiment. Blood samples were taken immediately before and once daily after the nephrectomy, at the beginning of the experiment, one hour afterward, and at the end. Samples of the ultrafiltrate were taken simultaneously with the blood collections during the filtration. Chlorides, urea N, creatinine, CO<sub>2</sub> capacity, proteins, glucose, and hematocrit were determined in some of these samples. The

\*The methods used were: urea nitrogen: Ormsby, A. A.: J. Biol. Chem. 146: 595, 1942; proteins: Weichselbaum, T. E.: Am. J. Clin. Path. 16: 40, 1946; creatinine: Folin, O., and Wu, H.: J. Biol. Chem. 38: 81, 1919; chlorides: Wilson, D. W., and Ball, E. G.: J. Biol. Chem. 79: 221, 1923; carbon dioxide capacity: Van Slyke, D. D., and Cullen, G. E.: J. Biol. Chem. 30: 289, 1917; blood volume: single samples in duplicate twelve minutes after injection of the dye (T1824); hematocrit: 10,000 r.p.m. during ten minutes in Winthrobe tubes.

These determinations were carried out in the Department of Biochemistry through the kindness of Dr. C. Cohn.

TABLE I. OBSERVATIONS ON MALE DOG, WEIGHT 27 POUNDS, RENDERED ANEPHRIC AND CONNECTED TO ULTRAFILTER\*

REMARKS		UREA N (MG./100 C.C.)	CREATININE (MG./100 C.C.)	PROTEIN (MG./100 C.C.)	CO <sub>2</sub> (VOL. %)	HEMATOCRIT (%)	Cl (MG./100 C.C.)
Preoperative	Blood	21		6.9	45	48	630
24 hr. after nephrectomy	Blood	65			32	47	680
48 hr. after nephrectomy	Blood	180		5.5	58	44	600
72 hr. after nephrectomy	Blood	175		5.4		38	540
After 1 hr. of filtration	Blood	135					590
	Plasma	145	8.6			42	590
After 8 hr. of filtration	Blood	75		3.9		39	640
	Plasma	83	6.0				600
Ultrafiltrate obtained at end of 1 hr.		160	7.2				660
Ultrafiltrate obtained at end of 8 hr.		80	5.0				660
		BEFORE FILTRATION			AFTER FILTRATION		
Plasma volume		964 c.c.			1010 c.c.		
Blood volume		1550 c.c.			1650 c.c.		
Hematocrit		38%			39%		

Total amount of filtrate, 7,200 c.c. collected in eight hr.

Total amount of urea N "excreted," 6.9 Gm.

Urea clearance (concentration in the blood assumed to be 125 mg./100 c.c., the mean between 175 and 75), 11.3 c.c./blood/minute.

\*In this experiment the lag seen in Fig. 9 and explained in the text has not been taken into consideration. This lag explains the lack of parallelism between the plasma and ultrafiltrate concentrations of urea and creatinine.

samples collected after one hour represent the results of the mixing with the donor's blood in the filter and the result of one hour of filtration. Fluid balance during the filtration was maintained by injecting intravenously every ten minutes an amount of Ringer-Krebs solution\* equal to the amount of ultrafiltrate collected during the interval.

In both types of experiment the inlet of the filter was connected with the central end of the femoral artery and the outlet with the central end of the femoral vein, the blood being circulated by means of the pump† previously described. The effective filtering pressure used was approximately 800 mm. of mercury. The dogs were injected with 40 to 50 mg. of heparin‡ immediately before the experiment to prevent blood coagulation and were narcotized with morphine, with a 1 per cent solution of procaine used locally.

In order to help avoid the formation of clots the cannulae used were as large as possible. They were washed with concentrated nitric acid before the experiment, cleaned with 4 per cent NaOH, and then rinsed in tap water. The system was filled with saline when only one or two filter tubes were used and with compatible dog's blood when more units were employed, in order to avoid air embolism and the possibility that the dog would lose too much blood into the filter. The blood was warmed to body temperature before it was returned to the animal.

\*The Ringer-Krebs solution was prepared by mixing the following substances in 100 c.c. of water: NaCl, 6.923 Gm.; KCl 0.354 Gm.; CaCl<sub>2</sub>, 0.057 Gm.; KH<sub>2</sub>PO<sub>4</sub>, 0.170 Gm.; MgSO<sub>4</sub>·H<sub>2</sub>O, 0.2925 Gm.; NaHCO<sub>3</sub>, 2.10 Gm.; Glucose, 1.0 Gm.; O<sub>2</sub> was bubbled through and the temperature was maintained at approximately 38° C.

†With a single filter tube the pump is not always necessary, as the normal arterial pressure is sufficient for the purpose.

‡Liquaemin liberally supplied to the department by Roche Organon, Inc., Nutley, N. J

*Results With Normal Dogs.*—Seven normal dogs were subjected to filtration with a single unit for periods ranging from one to five hours. The flow of blood through the filter was 50 to 70 c.c. per minute. All of the animals survived.

Uniformly, the amount of filtrate collected was 60 c.c. per hour, it was always protein-free, and in no case was hemolysis detected macroscopically. The concentration of urea in the blood and in the ultrafiltrate of one typical experiment is shown graphically in Fig. 9. As can be seen, there is a period of forty minutes during which the filtrate shows little urea concentration. This is due to the dilution of the ultrafiltrate by the saline held in the nylon tape. As this saline is replaced by ultrafiltrate, the urea concentration rises sharply and then levels off. The difference between the concentration of urea in the filtrate and that in the plasma may indicate that urea is not wholly diffusible or that it is bound. This possibility now is being investigated.

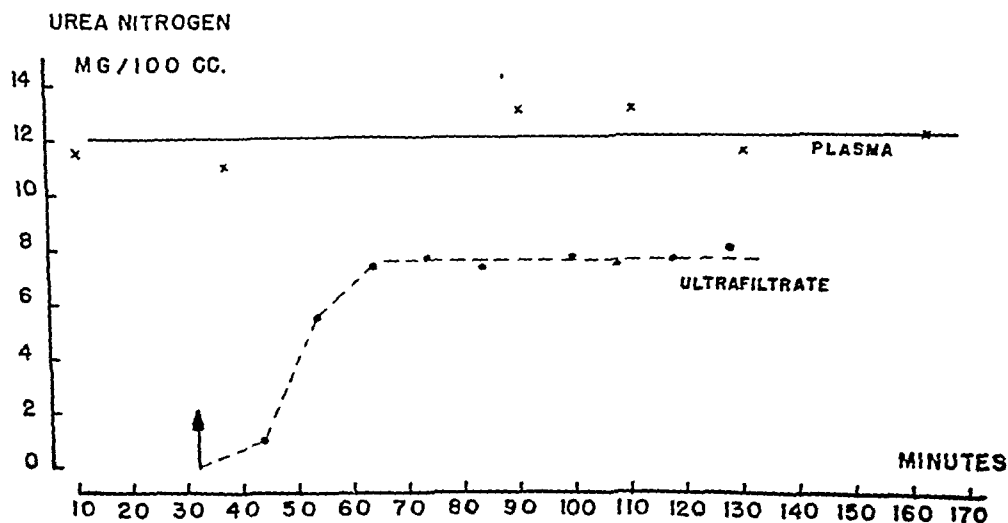


Fig. 9.—A typical experiment performed on a normal dog, showing the urea N concentration in the plasma and in the ultrafiltrate. The arrow indicates the beginning of filtration.

Several deductions can be drawn from these experiments:

1. The filter yields an appreciable amount of ultrafiltrate when connected with a dog.
2. The filter itself is not toxic. The dogs survived the experiment for a long time, and when they were sacrificed, autopsy revealed no macroscopic abnormalities. The limb used was in good condition despite the ligation of the femoral artery and vein.

*Results With Uremic Dogs.*—Four uremic dogs were used in the second type of experiment. The blood flow through the filter was 110 c.c. per minute with each. Two dogs died accidentally after two and three hours of filtration, respectively; the first died from shock due to blood loss, the second from pulmonary embolism. The other two dogs were filtered successfully for three and eight hours, respectively. They did not appear to be injured by the filtration,

judging from the general appearance and pulse rate, and were up and around when they came out of the anesthesia. These animals died in uremia thirty-six hours after the completion of the experiment. A typical result in one of these dogs is summarized in Table I.

These experiments show that the animals apparently were not adversely affected by the procedure, despite the fact that as much as 7,200 c.c. of fluid was filtered from the blood of a dog (and replaced by Ringer-Krebs solution) with a blood volume of 1,550 cubic centimeters. The chlorides returned to the normal level and the hematocrit remained constant during the experiment. Urea and creatinine were reduced by this ultrafiltration. Nevertheless, the fact that only 7 Gm. of urea were cleared in eight hours makes this procedure with the apparatus employed unsuitable for man, even though its use in dogs may considerably reduce the blood urea. A larger ultrafilter with more filtering surface, or one employing a much greater filtering pressure, may have a sufficient clearance of urea for man, but the dialyzer described by Kolff appears superior in simplicity and in obtainable results because the steeper gradient between urea concentration in the blood and the outside fluid dialyzed into seems to produce a greater urea clearance per unit area of cellophane than the ultrafiltration method described.

#### CONCLUSIONS

An ultrafilter employing cellophane is described and its applicability for experimental purposes noted.

The filter described has definite advantages over previous ones because of the following reasons:

1. It provides a large amount of ultrafiltrate in comparison with the volume of blood of the animal.
2. The blood in the filter can be placed under a wide range of pressures and can be circulated within the filter at different rates without the red blood cells being unduly traumatized. The filter does not lead to macroscopic hemolysis of the blood.
3. It is easily sterilized.

This ultrafiltration method, however, has limitations in its use for the control of uremia, especially since it has such low clearance values compared with the values reported by Kolff<sup>13, 14</sup> for dialysis.

Such a filter may be useful in the investigation of simultaneous clearances with the kidney and in the study of bound and unbound blood constituents. The composition of ultrafiltrates obtained during this investigation favors this opinion.

We wish to express our gratitude to Dr. L. N. Katz for his advice and guidance and to acknowledge the considerable assistance given us by other members of the department.

#### REFERENCES

1. Ferry, J. D.: Ultrafilter Membranes and Ultrafiltration, *Chem. Rev.* 18: 373, 1936.
2. Geiger, A.: Method of Ultrafiltration in Vivo, *J. Physiol.* 71: 111, 1931.
3. Laviates, P. H.: Anaerobic Ultrafiltration, *J. Biol. Chem.* 120: 267, 1937.

4. McBain, J. W., and Stuewer, R. F.: Ultrafiltration Through Cellophane of Porosity Adjusted Between Colloidal and Molecular Dimensions, *J. Phys. Chem.* 40: 115, 1936.
5. Kistler, S. S., and McBain, J. W.: Ultrafiltration as a Test for Colloidal Constituents in Aqueous and Nonaqueous Systems, *J. Phys. Chem.* 35: 130, 1931.
6. Pierce, H. F.: Nitrocellulose Membranes of Graded Permeability, *J. Biol. Chem.* 75: 795, 1927.
7. Seymour, W. B.: The Preparation of Cellophane Membranes of Graded Permeability, *J. Biol. Chem.* 3: 701, 1940.
8. Collidge, T. B.: A Simple Ultrafiltration Apparatus, *J. Biol. Chem.* 135: 541, 1940.
9. DeBakey, M.: Technique of Blood Transfusion; Description of Improved Continuous Flow Instrument and Needles, *J. Trop. Med.* 40: 137, 1937.
10. Achard, C.: Sur l'acide urique ultrafiltrable, *Compt. rend. soc. de biol.* 113: 658, 1932.
11. Bauer, J. H., and Hughes, T. P.: Preparation of Graded Collodion Membranes of Elford and Their Use in Study of Filterable Viruses, *J. Gen. Physiol.* 18: 143, 1934.
12. Brull, L.: Technique d'ultrafiltration avec membrane de cellophane, *Compt. rend. soc. de biol.* 120: 98, 1935.
13. Kolff, W. J., and Berk, H. T. J.: The Artificial Kidney; a Dialyser With a Great Area, *Acta med. Scandinav.* 117: 121, 1944.
14. Kolff, W. J.: Die Kunstmatige Niere, *J. H. Kok. N. V. Kampen, Holland*, 1946.



## BOOK REVIEWS

**Penicillin in Syphilis.** By Joseph Earle Moore, M.D., Associate Professor of Medicine and Adjunct Professor of Public Health Administration, the Johns Hopkins University; Physician-in-Charge, Syphilis Division of the Medical Clinic, and Visiting Physician, the Johns Hopkins Hospital; Chairman, Syphilis Study Section, National Institute of Health, United States Public Health Service; Chairman, Subcommittee on Venereal Diseases, National Research Council. Charles C. Thomas, Publisher, Springfield, Ill., 1946. Price \$7.50. Cloth with 731 pages, 109 illustrations, 122 tables.

The startling discovery of the treponemicidal action of penicillin by Mahoney, Arnold, and Harris in midsummer of 1943 resulted in the almost immediate organization of a nationwide cooperative study of the efficacy of this antibiotic in syphilis. This collaborative effort, which is perhaps unparalleled in medical history, was motivated by the urgent necessity of the Armed Forces for authoritative information regarding the effectiveness of this agent. Although the facilities of eight laboratories of experimental syphilis were enlisted for intensive study of penicillin in rabbit syphilis, time did not permit final definition of the time-dose relationship in the experimental animal before human trial was undertaken. Accordingly, clinical investigation has proceeded simultaneously in a total of forty-four clinics and treatment centers. Thus far, these participating agencies have treated some 25,000 patients with early syphilis by assigned schedules (thirty-six all told) in a uniform manner, results being analyzed as a group by a central statistical unit.

Since the outset of the study Moore in his capacity as Chairman, first of the Penicillin Panel of the National Research Council and more recently of the Syphilis Study Section of the United States Public Health Service, the sponsoring organizations, has occupied a position uniquely fitting him for authorship of this monograph. As a consequence, he has had access to material restricted during the war years, some of which still is unreported, as well as to unpublished data of the individual cooperating groups. The author thus has been able to assemble under one cover all the information relating to the effect of penicillin in syphilis available as of October, 1946.

As stated in the preface, "The author's only excuse (for this monographic presentation of the admittedly preliminary results now available\*) can be that so far, the medical profession has been informed only that penicillin is of value in syphilis. How best to employ it in the several stages of syphilitic infection, on the basis of current knowledge, has nowhere yet been systematically presented. This monograph is an effort to supply the deficiency. It is prepared with full knowledge of its inadequacies and of the necessity for frequent revision to maintain it up to date."

The material is presented in nineteen chapters—actually eighteen, since one (the last) is concerned with streptomycin. Of these, the first seven are devoted to fundamental information—the chemistry of the penicillins, pharmacology, toxicity, therapeutic efficacy, etc. For the student of syphilis the author's exposition of these basic data is unquestionably of greater value than the chapters concerned with clinical use. Of particular interest is the discussion of the results obtained by the cooperating laboratories in experimental syphilis and the implications of these results as to the mode of action of penicillin against *Treponema pallidum*. The experimental data thus far available suggest, and this is not belied by the accumulating clinical results, that the *desideratum* in syphilis may not be large dosages over short periods of time but smaller dosages prolonged for weeks or perhaps even months. If these indications are borne out by further study, the hope of a quick, safe (that is, with penicillin alone) cure for early syphilis suitable for mass application may prove to be unattainable.

\*Parenthetical words are the reviewer's.

One chapter is concerned with the administration of penicillin for other diseases as a source of confusion in the diagnosis of syphilis. The suppressing effect of penicillin administered for the treatment of gonococcal infections on the early manifestations of syphilis is emphasized, as is the necessity for prolonged serologic follow-up (at least four months) of penicillin-treated patients with gonorrhea, to rule out simultaneously acquired syphilis.

The remaining ten chapters recount the accumulated information pertaining to the clinical use of penicillin in the various stages of syphilitic infection. Since, from the standpoint of the Army and Navy during the war years, early syphilis and the various types of late neurosyphilis presented the greatest therapeutic problems, emphasis has been directed toward defining the place of penicillin in the treatment of these stages of the disease. The early syphilis results are those accumulated prior to August, 1945, based on the treatment of approximately 11,000 patients by twenty-six treatment regimens. At the time this book was written, patients treated since the date mentioned had been followed too short a period of time for even preliminary evaluation. The results of penicillin treatment of early syphilis may be summarized with the statement that the optimum regimen of treatment with penicillin (sodium or calcium suspended in peanut oil and beeswax), either alone or combined with metallothérapie, still is not defined since the over-all cumulative failure rates of even the best schedules approximate 15 per cent at the end of one year. Suggested schedules utilizing larger and/or longer dosage schedules than those so far analyzed are appended by the author who implies, though it is not so stated, that the results may be no better than with smaller amounts.

Scattered information based on small series of patients indicates that penicillin is effective in early syphilis resistant to arsenic and bismuth, in benign late syphilis, and in at least one type of ocular involvement (the *iritis* of early syphilis); however, in others such as interstitial keratitis and primary optic atrophy, results are only partially successful. In view of the absence of serious toxic reactions few will disagree that penicillin is the treatment of choice of the pregnant woman with syphilis and probably also of the infant with early congenital syphilis. Little or no information is available as to the efficacy of penicillin in either latent or cardiovascular syphilis since these are both long-term projects. In this respect, however, the physician is well advised that penicillin is no more effective than combined arsenical and bismuth therapy in rendering the blood test negative, since in late syphilis seroresistance is the rule regardless of the agent employed.

The discussion of penicillin in neurosyphilis reflects more than elsewhere the author's own experience, past as well as present, in the treatment of these complex syndromes. The results with penicillin, even though preliminary, demonstrate conclusively that this agent exerts a profound effect on the spinal fluid abnormalities, the improvement in these elements continuing for many months after treatment. At the present time major discussion centers around the question of whether penicillin plus fever offers any advantage over penicillin alone. Comparative data are scanty, and, although the trends of the spinal fluid syndromes presented by the author suggest that combined treatment may be slightly superior, the results for the most part are not statistically significant. Until more evidence has been accumulated many syphilotherapists will continue to regard the superiority of combined penicillin and fever as not proved; but few will disagree that for the present the combination should be recommended for the graver parenchymatous forms of neurosyphilis. There undoubtedly will be dissenters to the author's opinion that induced malaria holds any advantage over fever therapy by mechanical means.

Sufficient has been said to indicate that Moore has presented in detail the available information about the effect of penicillin in syphilis. It is to be hoped that this book will serve to correct many misconceptions regarding the use of penicillin in this disease and the results to be expected therefrom. It was inevitable that, in a few respects at least, the book would be outdated already on the day of publication. This fact does not detract from the vast fund of basic data that the book presents, and this reviewer, for one, hopes that the author's promise of frequent revision will be fulfilled.

VIRGIL SCOTT.

**Diseases of the Retina.** By *Herman Elwyn, M.D.*, Senior Assistant Surgeon, New York Eye and Ear Infirmary. The Blakiston Company, Philadelphia, 1946. Price \$10.00. Cloth with 587 pages, 170 illustrations, 19 in color.

The author has brought up to date the clinical picture of retinal diseases, correlating the abnormal physiology with the subjective and objective findings of the subject. The course of each disturbance is followed through the life picture of the disease. Treatment is discussed concisely from the viewpoint of the ophthalmologist, who is familiar with the closely associated fields of internal medicine and neurology.

The book is divided into eight parts: I. Diseases of the Retina Resulting From Disturbances in Circulation; II. Diseases of the Retina Resulting From Vascular Malformations; III. Degenerative Diseases of the Retina on a Hereditary Basis; IV. Inflammatory Diseases of the Retina; V. Tumors of the Retina; V. Diseases of the Retina Leading to Retinal Detachment; VII. Developmental Anomalies of the Retina; VIII. Radiation Injuries of the Retina.

The present-day concept of vascular disease per se, along with the primary vascular change occurring in inflammatory, metabolic, and degenerative diseases, has been handled extremely well. The author has followed a definite plan in presenting his material in an orderly and concise manner with the minimum expenditure of words.

This volume belongs in the library of all ophthalmologists, internists, and neurologists.

WILLIAM M. JAMES, M.D.

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## ANNOUNCEMENT

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### REVISED BIOLOGICS REGULATIONS

It is desired to call attention to a revision of the regulations governing the manufacture and sale of biologic products published in the Federal Register on Jan. 21, 1947, and effective thirty days after that date.

As is indicated in these regulations, the scope of the Biologics Law is broadened somewhat, and certain diagnostic products administered or applied to a person or prepared from or with the aid of a biologic product now should be distributed in interstate commerce only if prepared by a licensed laboratory.

Also requiring a license for distribution are blood grouping and Rh typing serums, for which minimum requirements have been prepared in anticipation of the revision of the regulations.

Information as to the status of any of these products, or the requirements applicable to them, may be obtained from the Biologics Control Laboratory, National Institute of Health, Bethesda 14, Md.

## MECHANISM OF THE THYMOL TURBIDITY TEST

PHILIP P. COHEN, M.D., AND FRANCES L. THOMPSON, B.S.  
MADISON, WIS.

THE thymol turbidity test of Maclagan has been shown to be of considerable diagnostic value in certain types of liver disease.<sup>1-4</sup> Maclagan<sup>1</sup> and Carter and Maclagan<sup>4</sup> have reported that the test was probably associated with an increase in gamma globulin. Recant and associates,<sup>5</sup> on the other hand, have shown that the test depended on the presence of a lipid-containing fraction of serum globulin rather than the gamma globulin.

In the present study experiments are reported which show that the thymol turbidity test depends on a reaction involving chiefly the beta globulin fraction of serum.

### METHODS AND PROCEDURES

*Thymol Reagent and Test.*—The thymol reagent was prepared essentially as recommended by Maclagan.<sup>1</sup> It was found that the reagent developed a turbidity on standing after seven to ten days. However, reheating, cooling, and again seeding with thymol crystals resulted in a clear solution.

Thymol turbidity values were estimated from a set of Kingsbury<sup>6</sup> visual standards calibrated against known amounts of human serum albumin and sulfosalicylic acid.

*Treatment of Serum With Thymol Reagent for Electrophoretic Analysis.*—An aliquot of serum, usually 10 c.c., was diluted with two parts of thymol buffer, pH 7.8, and dialyzed in the cold room (2° C.) against 200 to 250 c.c. of thymol buffer, pH 7.8, for twelve hours or longer. At the end of the dialysis period the precipitate was removed by centrifugation.

The crystal clear supernatant (thymol-treated serum is consistently more translucent than untreated serum) was then dialyzed in the cold room twice against 200 c.c. of 0.1  $\mu$  sodium diethylbarbiturate buffer, pH 8.6, and finally against 1,500 c.c. of the same buffer for at least thirty-six hours. The precipitate was resuspended in a volume of 0.1  $\mu$  barbiturate buffer, pH 8.6, to give a protein concentration of between 0.5 and 1.0 per cent. The resulting turbid solution was dialyzed in the cold room against several changes of buffer and finally against 1,000 c.c. of buffer for at least thirty-six hours. High speed centrifugation (10,000 r.p.m.) for ten minutes was found effective in clarifying the solution.

*Electrophoretic Analysis.*—Electrophoresis was carried out at a potential gradient of about 6 volts per centimeter, at 1.5° C., using the schlieren scanning

From the Laboratory of Physiological Chemistry, University of Wisconsin Medical School. Supported in part by a grant from the Wisconsin Alumni Research Foundation. Received for publication, March 27, 1947.

technique of Longsworth.<sup>7</sup> Most of the runs were made with the single center section 11 c.c. cell. A micro cell, 2 c.c. capacity, was employed in some instances for both the thymol precipitate and the treated and untreated serum samples. A comparative study of the patterns and mobilities of the same serum with the macro and micro cells revealed close agreement in a series of ten determinations.

Relative composition of the serum samples was estimated by measuring the areas under the projected patterns after the areas were resolved into a series of symmetrical curves. In all instances the percentage composition represents the average of the ascending and descending boundaries. Mobilities were de-

TABLE I. ELECTROPHORETIC COMPOSITION OF SERUM BEFORE AND AFTER TREATMENT WITH THYMOL REAGENT

SERUM	DIAGNOSIS	THYMOL TURBIDITY UNITS	TOTAL PROTEIN (GM./100 C.C.)	PER CENT COMPOSITION				
				ALBU- MIN	ALPHA 1	ALPHA- 2	BETA	GAMMA
1A*	Laënnec's cirrhosis	4	5.8	32.6	7.3	7.6	17.0	35.5
1B*				41.1	7.0	12.3	7.5	32.1
2A	Infectious hepatitis	10	7.1	40.7	6.2	12.9	19.0	21.2
2B				49.6	7.4	15.0	8.5	19.5
3A	Infectious hepatitis	9	7.2	45.5	4.5	9.7	18.4	21.9
3B				51.8	5.0	10.6	12.6	20.0
4A	Infectious hepatitis	14	8.1	39.8	5.6	10.6	16.4	27.6
4B				40.8	5.7	8.9	13.3	31.3
5A	Biliary cirrhosis	8	6.0	27.3	4.7	8.2	15.0	44.8
5B				34.5	4.6	9.2	8.0	43.7
6A	Infectious hepatitis	10	7.8	43.7	4.9	9.0	17.9	24.5
6B				54.7	5.6	10.5	6.5	22.7
7A	Infectious hepatitis	6	8.3	40.0	5.4	10.6	15.6	28.4
7B				45.1	5.9	10.9	9.2	28.9
8A	Chronic hepatitis	4	7.2	51.3	3.8	8.4	18.2	18.3
8B				58.5	5.0	8.4	12.4	15.7
9A	Infectious hepatitis	4	6.8	51.8	4.6	10.0	19.9	13.7
9B				55.7	6.3	10.4	12.4	15.2
10A	Infectious hepatitis	8	7.2	53.6	4.2	8.6	16.2	17.4
10B				60.3	5.0	12.3	7.6	14.8
11A	Chronic hepatitis; renal tuberculosis	10	5.0	43.6	5.8	10.0	15.9	24.7
11B				50.3	6.9	10.9	7.7	24.2
11C*				50.7	8.0	12.3	6.3	24.0
Average A				42.7	5.2	9.6	17.2	25.3
Average B				48.4	5.7	10.9	9.6	24.4
12A	Normal	0	7.1	56.7	6.8	9.0	13.2	14.3
12B				59.0	6.2	9.2	11.7	13.9
12C				58.1	8.6	8.9	10.2	14.2
Electrophoretic Analysis of Ten Sera From Normal Adults								
Mean				56.5	5.2	9.0	15.2	14.2
Standard deviation				2.8	1.7	1.4	1.5	1.8
Standard error of mean				0.9	0.6	0.5	0.5	0.6

\*Sample A refers to untreated serum; sample B refers to serum after dialysis for twelve hours against thymol reagent and removal of precipitate; sample C refers to serum after dialysis for seventy-two hours against thymol buffer and removal of precipitate.

terminated by measuring the migration distances from the salt boundaries. Conductivity measurements were made on the protein-free buffer used as the dialyzing solution.

### RESULTS

The percentage composition of serum before and after thymol treatment is shown in Table I. In each instance the thymol turbidity value is indicated. The most striking change in the composition of the serum after treatment with thymol reagent is the decrease in the beta globulin fraction. As can be seen,

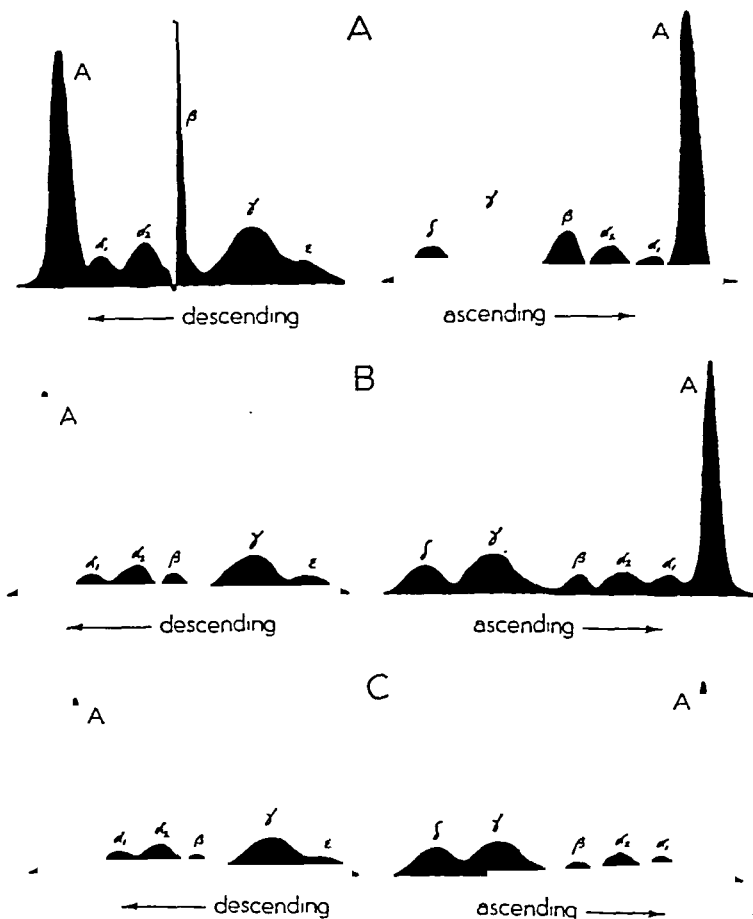


Fig. 1.—Electrophoretic diagrams of Serum 11 (Table I) before treatment with thymol reagent (A), after treatment for twelve hours (B), and after treatment for seventy-hours (C).

this fraction is decreased in all instances including the normal serum although the decrease in the latter case is small. Treatment of a thymol-negative and a thymol-positive serum for seventy-two hours instead of the usual twelve hours with thymol reagent resulted in a small additional decrease in the beta globulin percentage (Serum 11C and 12C, Table I). The average decrease in the beta globulin percentage as a result of thymol treatment is of the order of 50 per

cent. The average decrease in the percentage of gamma globulin is of the order of 4 per cent. All the other fractions show an increase, the albumin percentage being increased the most. It is apparent from these data that the beta globulin fraction is the only fraction of serum showing a striking change as a result of thymol treatment. The electrophoretic diagrams of Serum 11 (Table I) before and after treatment with thymol reagent are seen in Fig. 1. The disappearance of the beta anomaly after thymol was seen in all instances.

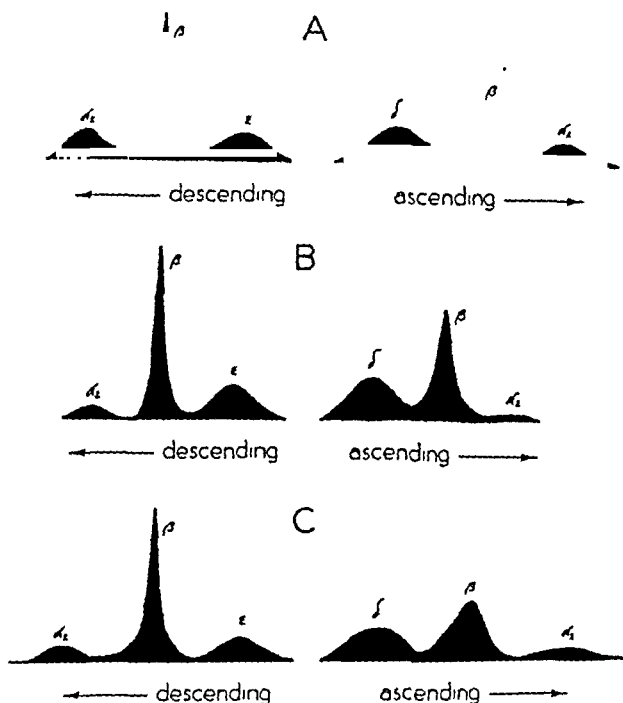


Fig. 2.—Electrophoretic diagrams of thymol precipitates. *A*, From normal (Serum 12, Table I); *B*, chronic hepatitis (Serum 11, Table I); *C*, mixture of precipitates *A* and *B*.

Further evidence in support of the role of beta globulin is seen in the electrophoretic diagrams of the thymol precipitates (Fig. 2). Diagram *A* of Fig. 2 is that of the thymol precipitate from normal Serum 12 (Table I); diagram *B* is that from Serum 11 (Table I); diagram *C* represents an equal mixture of thymol precipitate from Sera 11 and 12 (Table I). From the diagrams it is apparent that the thymol precipitate consists of one major component which has a mobility of a beta globulin (Table II) and a smaller and irregularly appearing component which has a mobility of an alpha-2 globulin. It is of interest to note that the descending patterns of the thymol precipitates have the appearance of a beta anomaly. When the thymol precipitates of a normal and pathologic serum are mixed (diagram *C*, Fig. 2), the components have the same mobility and composition as the unmixed precipitates. The mobilities of the various components of serum before and after thymol treatment, of the precipitates, and of normal serum are shown in Table II.

TABLE II. MOBILITIES (CM.<sup>2</sup>/VOLT SEC.  $\times 10^{-5}$ )

	ALBUMIN	ALPHA-1	ALPHA-2	BETA	GAMMA
Normal					
Mean	6.92	5.90	4.70	3.42	1.64
S.D.	0.23	0.25	0.20	0.28	0.29
Liver disease					
Before thymol					
Mean	6.95	6.1	4.86	3.63	1.70
S.D.	0.35	0.32	0.22	0.25	0.27
After thymol					
Mean	6.95	6.0	4.92	3.75	1.72
S.D.	0.31	0.30	0.31	0.33	0.32
Thymol precipitate					
Mean	—	—	—	3.30	—
S.D.	—	—	—	0.32	—

## DISCUSSION

It is obvious from the data presented that thymol reagent reacts chiefly if not exclusively with the beta globulin fraction of serum. The fact that the beta globulin levels in the thymol turbidity-positive cases are not strikingly higher than those observed with a series of normals (Table I) suggests that this fraction has an abnormal component. This is further supported by the fact that treatment of normal serum with thymol reagent results in only a small decrease in the beta globulin level (Serum 12, Table I). The precipitate, however, is indistinguishable, electrophoretically, from that obtained from pathologic serum (diagrams A and C, Fig. 2).

It is of some interest to compare the electrophoretic patterns of sera of this series of liver diseases with those from normal adults. If the average values of the pathologic sera are compared with the average values of the normal series (Table I), it will be noted that in the former group the albumin is decreased, the beta globulin is increased slightly, and the gamma globulin is strikingly increased. It should be noted also that while the gamma globulin level is increased above the normal value in all cases but Serum 9, there is no correlation between the gamma globulin increase and the thymol turbidity units. A similar lack of correlation, however, is also to be seen in the case of the beta globulins.

The known complexity of the beta globulin fraction makes any attempt at a discussion of the possible nature of the changes in this fraction in disease difficult. It is possible that there is no fundamental change in the protein components making up this fraction, but rather a change in either the kind or amount of phospholipid chemically bound with the protein. While most patients with nonobstructive liver disease do not show any consistent increase in phospholipid content of serum,<sup>10</sup> it is possible that some alteration in the amount or type of true lipoprotein rather than total lipid is involved.

A sample of the thymol precipitate from Serum 11 was examined in the ultracentrifuge. The material had the same number and kind of components as are seen with the beta globulin fraction obtained as a by-product of fractionation of gamma globulin from normal human plasma.<sup>8</sup> This normal beta globulin fraction was approximately 90 per cent homogeneous electrophoretically.<sup>9</sup> Thus it would appear that on the basis of the properties in the electrophoresis



apparatus and in the ultracentrifuge, there are no physical differences to be noted between the normal and pathologic beta globulin fractions.

#### SUMMARY

1. Electrophoretic analyses of sera from patients with liver disease having positive thymol turbidity tests were made before and after treatment with thymol reagent. The chief change noted was a decrease of the beta globulin fraction of the supernatant after removal of the thymol precipitate.

2. The thymol precipitates obtained from sera from both normal and diseased cases were examined in the electrophoresis apparatus and both were shown to migrate with the mobility of a beta globulin. Examination in the ultracentrifuge revealed the thymol precipitate to have a composition similar to that seen with the beta globulin fractions obtained from normal human plasma.

3. The possible basis for the thymol turbidity test is briefly discussed.

#### REFERENCES

1. Maclagan, N. F.: The Thymol Turbidity Test as an Indicator of Liver Dysfunction, *Brit. J. Exper. Path.* 25: 234, 1944.
2. Shank, R. E., and Hoagland, C. L.: A Modified Method for the Quantitative Determination of the Thymol Turbidity Reaction of Serum, *J. Biol. Chem.* 162: 133, 1946.
3. Watson, C. T., and Rappaport, E. M.: A Comparison of the Results Obtained With the Hanger Cephalin-Cholesterol Flocculation Test and the Maclagan Thymol Turbidity Test in Patients With Liver Disease, *J. LAB. & CLIN. MED.* 30: 983, 1945.
4. Carter, A. B., and Maclagan, N. F.: Some Observations on Liver Function Tests in Diseases Not Primarily Hepatic, *Brit. M. J.* 2: 80, 1946.
5. Recant, L., Chargaff, E., and Hanger, F. M.: Comparison of the Cephalin-Cholesterol Flocculation With the Thymol Turbidity Test, *Proc. Soc. Exper. Biol. & Med.* 60: 245, 1945.
6. Kingsbury, F. B., Clark, C. P., Williams, G., and Post, A. L.: The Rapid Determination of Albumin in Urine, *J. LAB. & CLIN. MED.* 11: 981, 1926.
7. Longworth, L. G.: Recent Advances in the Study of Proteins by Electrophoresis, *Chem. Rev.* 30: 323, 1942.
8. Deutsch, H. F., Alberty, R. A., and Gosting, L. J.: Biophysical Studies of Blood Plasma Proteins. IV. Separation and Purification of a New Globulin From Normal Human Plasma, *J. Biol. Chem.* 165: 21, 1946.
9. Deutsch, H. F.: Personal communication.
10. Man, E. B., Kartin, B. L., Durlacher, S. H., and Peters, J. P.: The Lipids of Serum and Liver in Patients With Hepatic Diseases, *J. Clin. Investigation* 24: 623, 1945.

## A STUDY OF THE SERUM BILIVERDIN CONCENTRATION IN VARIOUS TYPES OF JAUNDICE

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THE presence of a green tinge in the skin of certain patients with jaundice has long been recognized. The relation of this so-called biliverdin jaundice to the underlying disease has been discussed by various writers.<sup>6, 7, 10, 14</sup>

According to recent chemical evidence,<sup>2, 3, 11, 18-23, 31</sup> it is probable that biliverdin is intermediate in the catabolism of hemoglobin to bilirubin. Demonstration by Rich<sup>27, 28</sup> of both biliverdin and bilirubin in the phagocytes of tissue cultures of mesothelial origin, the occurrence of biliverdin in the Kupffer cells of geese as observed by McNee,<sup>25</sup> and its presence in dog's placenta<sup>18</sup> and hematoma fluid<sup>16</sup> emphasize a close physiologic connection between biliverdin and hemoglobin breakdown. The "green hemoglobins" studied by Fischer,<sup>11</sup> Warburg,<sup>31</sup> Lemberg,<sup>18-23</sup> Barkan,<sup>2, 3</sup> and their co-workers, to which the name "verdo-hemochromogen" was given by Lemberg,<sup>20</sup> are undoubtedly biliverdin-iron-protein complexes. Assuming that biliverdin is an intermediate stage in the transition of hemoglobin to bilirubin, its reduction to the latter substance in the body normally proceeds in a manner that excludes its appearance in the serum or plasma in evident amounts.

Methods have been described for the determination of biliverdin in bile,<sup>1, 12</sup> but these are not suitable for blood serum or plasma. Biliverdin does not give a diazo reaction and, thus, is not included in the quantitative van den Bergh method for bilirubin, regardless of modification. Specific color reactions for biliverdin are unknown. The purpose of the present study was to develop a method for the quantitative determination of biliverdin in serum or plasma and to apply this method in studying various patients with jaundice, especially those exhibiting greenish or outspoken green tints.

### METHODS

Crystalline biliverdin was prepared by the addition of purified bilirubin\* to boiling glacial acetic acid containing ferric chloride, according to Lemberg's method.<sup>17</sup> The dry crystalline substance was weighed on a microbalance, and a 0.4 mg. per cent solution in 0.01 per cent sodium hydroxide was prepared. Employing the Evelyn photoelectric colorimeter, a transmission curve then was determined for this solution. A like curve was obtained for a similarly prepared solution of bilirubin. These curves are shown in Fig. 1. Inspection establishes the suitable wave length for measurement of biliverdin between 635 and 690 millimicrons. Between these two points bilirubin transmits well, whereas biliverdin has a relatively great absorption. Thus, the 660 m $\mu$  filter was employed in the present study.

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The use of direct regional spectrophotometry in this determination necessitates exclusion of the presence of appreciable quantities of reduced and oxy-hemoglobin, methemoglobin, methemalbumin, hematin, and sulfhemoglobin. These interfering pigments, in contrast to biliverdin, absorb more light in the region of 620 to 635  $m\mu$  than at 660; initial comparison in these two regions thereby serves to indicate their presence. Fortunately the interfering pigments are seldom encountered in carefully manipulated unhemolyzed specimens, though the presence of any appreciable quantity of any one substance casts doubt upon the results of the biliverdin determination. Furthermore, the use of nonturbid, fasting sera is obviously necessary. The reader is referred to the work of Drabkin and Austin<sup>8</sup> for further orientation concerning the light absorption of the various forms of hemoglobin.

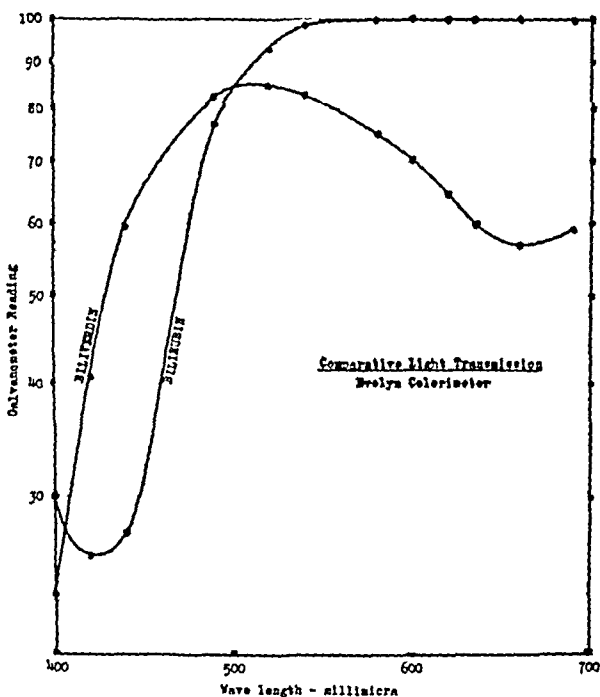


Fig. 1.—Transmission curves for solutions of crystalline bilirubin and biliverdin.

A calibration curve was determined as follows: One cubic centimeter quantities of clear, fasting normal sera, containing varying known quantities of added crystalline biliverdin in solution, were added to 8 c.c. quantities of distilled water and mixed. The resultant turbidity was then dispelled by the addition of 1 c.c. of saturated sodium chloride solution. Utilizing the Evelyn colorimeter, the 660  $m\mu$  filter, and a predetermined center setting (see next paragraph), a straight-line relationship was observed when the galvanometer readings were plotted against concentration on semilogarithmic paper (Fig. 2).

Normal sera, containing no biliverdin as evidenced by no dip in transmission between 620 and 660  $m\mu$ , even when they appear to be clear, may give

galvanometer readings between 98 and 100 when a blank (containing 9 c.c. of water and 1 c.c. of saturated sodium chloride) is set at 100. Consequently, a center setting of the instrument is determined from a diluted normal serum which, after giving a reading of 98 against the theoretical blank, is then set to read 100. This precaution prevents the apparent measurement of biliverdin when actually none is present, but conversely, very small amounts, less than 0.02 mg. per cent may be missed.

The serum bilirubin was determined by the method of Malloy and Evelyn,<sup>24</sup> modified in accordance with the recommendation of Ducci and Watson.<sup>9</sup>

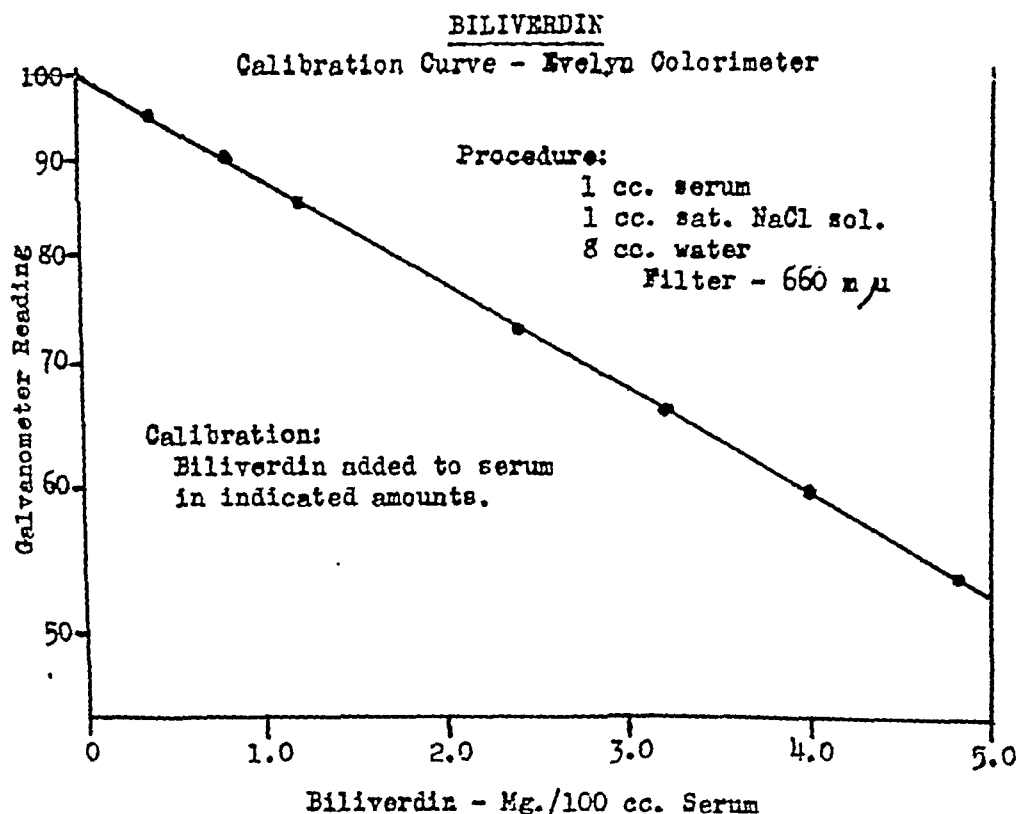


Fig. 2.—Calibration curve obtained by adding varying amounts of crystalline biliverdin to normal serum.

#### RESULTS

The sera of twelve nonjaundiced individuals, including both hospital patients and normal healthy persons, were examined for biliverdin. None was found in this series by the method described. Values ranging from zero to 2.2 mg per cent were obtained in the course of 105 observations on the sera of sixty-six patients with jaundice.

Significant levels of serum biliverdin were found in the following patients: twenty-seven with carcinomatous obstruction of the common bile duct (Fig. 3), five with infectious hepatitis (Fig. 4), five with cirrhosis and jaundice

TABLE I. PATIENTS WITH JAUNDICE WITH NO BILIVERDIN IN SERUM

PATIENTS	CONDITION	BILIRUBIN	
		DIRECT TOTAL	TOTAL Mg. %
6	Transfusion reaction	.11	5.4
		.08	4.6
		--	11.0
		--	3.0
		.83	30.0*
3	Hemolytic anemia	.62	3.4
		.17	3.6
		.19	5.0
		.66	14.1
1	Infectious mononucleosis	.78	3.2
1	Myelogenous leucemia	.16	3.3
1	Hodgkin's disease	.22	11.3
1	Banti's disease	.31	9.3
1	Cardiac Failure	.52	5.5
		.42	4.0

\*Questionable biliverdin.

(Fig. 4), and five with common bile duct stenosis (Fig. 5). Among these were fourteen individuals with a total bilirubin under 10 mg. per cent (average, 5.9 mg. per cent). On the other hand, of ten with calculous obstruction of the common duct, five had no biliverdin in the serum, although their average total bilirubin was 7.1 mg. per cent (Fig. 5).

The remaining fourteen patients (Table I) with a range of total bilirubin from 3 to 30 mg. per cent (average 7.6 mg. per cent), twelve of whom had

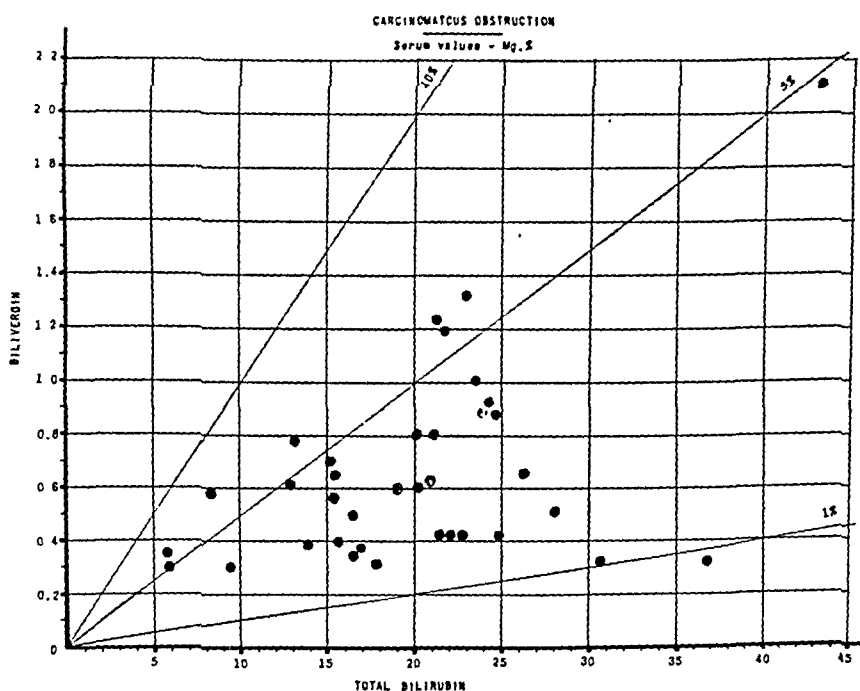


Fig. 3.—Serum biliverdin values in twenty-seven patients with carcinomatous obstruction of the common bile duct plotted against the total serum bilirubin (per cent biliverdin of total bilirubin represented by the radiating lines).

either a hemolytic process or bilirubinemia of the predominantly indirect type, all had zero biliverdin. The interference of methemalbumin in the method was not recognized early in the study but undoubtedly accounts for the slight galvanometer deflection in the patient marked by the asterisk (Table I), in which a definite in vivo hemolysis was known to have occurred.

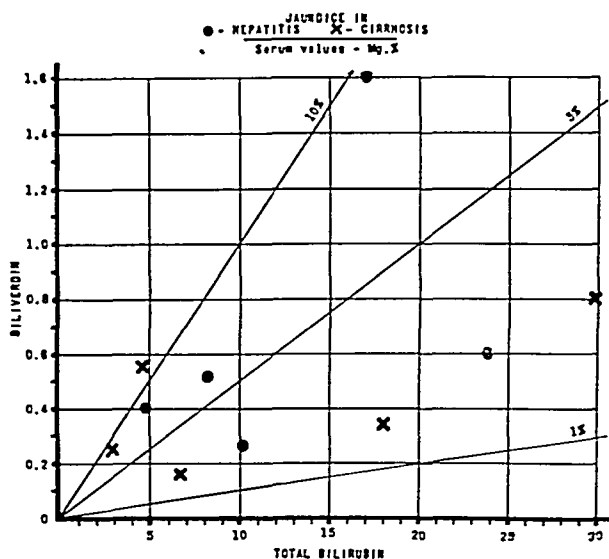


Fig. 4.—Serum biliverdin values plotted against total serum bilirubin in patients with infectious hepatitis and cirrhosis.

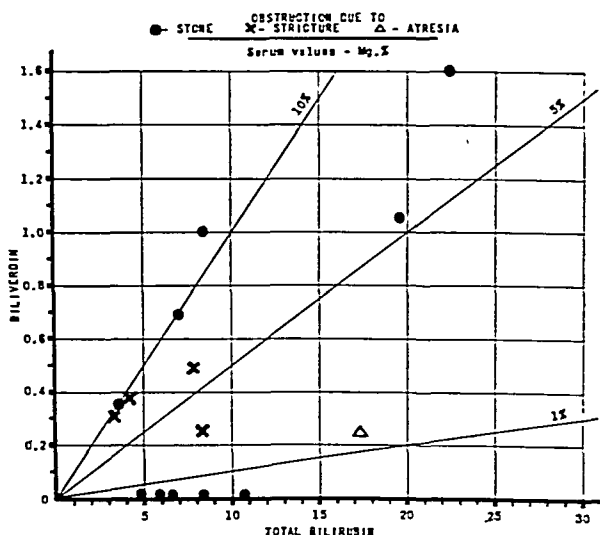


Fig. 5.—Serum biliverdin and total bilirubin values in patients with stone, stricture, and atresia of the common bile duct.

A variation in biliverdin concentration without respect to the direction of change in total bilirubin was noted during serial observations on twelve patients (Figs. 6 and 7); of these, the three in Fig. 7 stood out as being the

only ones who had received food or intravenous feeding to raise their caloric intake above basal requirements, resulting in a weight gain in two instances. In these three biliverdinemia disappeared completely despite the absence of any marked change in the total serum bilirubin.

A green tint was observed in the skin of patients having as little as 0.3 mg. per cent in the serum.

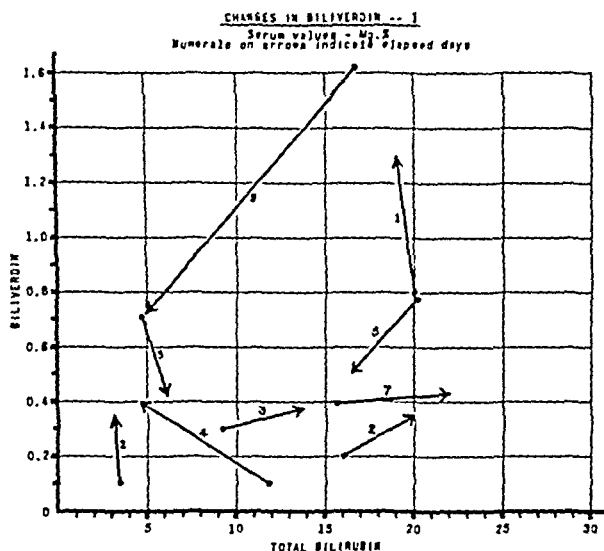


Fig. 6.—Changes in the serum biliverdin and bilirubin concentrations in serial studies.

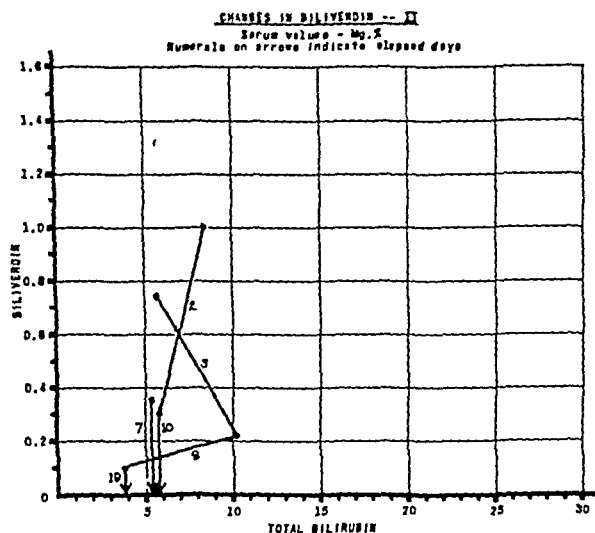


Fig. 7.—Disappearance of biliverdinemia without corresponding change in total serum bilirubin in patients with notably high caloric intakes.

#### DISCUSSION OF RESULTS

Lemberg and Wyndham<sup>21</sup> demonstrated a rapid, *in vitro* transformation of biliverdin to bilirubin in the presence of liver, kidney, and brain tissue and a

slower change in the presence of other tissues. They also observed a more rapid and complete transformation by the liver tissue of well-fed animals as compared with that of starved animals; furthermore, the addition of glucose to the tissue preparations from the latter animals accelerated the change. Lemberg<sup>22</sup> further emphasized the role of the liver in the reduction of biliverdin to bilirubin and the particularly close connection between the metabolism of bile pigment and carbohydrate in the liver.

The latter hypothesis gains possible support from the experience of Kanasaki,<sup>15</sup> in which green bile in the duodenal drainage of a patient with cirrhosis was replaced by orange bile after administration of intravenous glucose. Furthermore, biliverdin rather than bilirubin is excreted in the bile of starving dogs<sup>13</sup> and by frogs<sup>26</sup> whose livers are low in glycogen content. The oxidation-reduction possibilities which may exist in the liver and biliary duct system, both in health and disease, have been discussed by a number of investigators.<sup>4, 5, 22, 23, 29, 30</sup>

In the present study the trend was for the higher values of serum biliverdin to occur in patients with the higher values for total bilirubin, though notable exceptions occurred. In agreement with the findings of Eppinger,<sup>10</sup> the results indicate that the most pronounced biliverdin icterus and the highest values for serum biliverdin were associated with neoplastic obstruction of the common bile duct. However, biliverdinemia was a feature of other types of regurgitation jaundice as well, especially infectious hepatitis. The bulk of evidence favors a failure of change in biliverdin to bilirubin in the liver and/or other tissues. Noteworthy in this respect is the lack of biliverdinemia in the patients with hemolytic jaundice in which there was an obvious rapid transformation of blood to bile pigment. As to causes of biliverdinemia other than a failure of the liver or other organs and tissues to reduce biliverdin to bilirubin, the spontaneous oxidative formation of biliverdin from bilirubin in the gall bladder and bile ducts, with subsequent reabsorption, must be considered. In this regard, the patients with a high biliverdinemia did not show greater clinical evidence of infection than those with equal jaundice and less biliverdinemia. Furthermore, serial observations in twelve patients indicated that the serum biliverdin concentration can change markedly in one to five days without corresponding change in total bilirubin and, therefore, in the case of common duct obstruction, without change in the reabsorption possibilities from the biliary tree.

In contrast to the clinical observations of Brugsch<sup>6</sup> and Horsters,<sup>14</sup> biliverdinemia was not observed in jaundice due to hemolytic processes.

#### SUMMARY AND CONCLUSIONS

1. A direct spectrophotometric method for the quantitation of biliverdin in serum or plasma is described.
2. Observations on sixty-six patients with jaundice due to various causes are summarized. In general, biliverdinemia is a feature of regurgitation jaundice; it has not been observed in hemolytic (retention) jaundice. Absence of biliverdinemia speaks against a diagnosis of jaundice due to neoplastic obstruction.



3. There is suggestive evidence that biliverdinemia is abolished in jaundice when the nutritional status of the patient improves.

## REFERENCES

1. Amada, Y.: Versuche über die quantitative Erfassung der Verdinanteile der Gallenfarbstoffe in der Galle, *J. Biochem.* 32: 187, 1910.
2. Barkan, G.: Blutfarbstoff, Eisen, Gallenfarbstoff; neuere Untersuchungen über eisenhaltige Begleiter des Hämoglobins, *Klin. Wchnschr.* 16: 1265, 1937.
3. Barkan, G., and Schales, O.: Chemischer Aufbau und physiologische Bedeutung des "leicht abspaltbaren" Bluteisens. 13. Mitteilung in der Reihe der Eisenstudien, *Ztschr. f. physiol. Chem.* 248: 96, 1937.
4. Barry, H. M., and Levine, V. E.: The Oxidation and Reduction of Bile Pigments, *J. Biol. Chem. (Proceedings)* 59: lii, 1924.
5. Baumgärtel, T.: Zur Kenntnis der biologischen Erythrocytolys, *Klin. Wchnschr.* 22: 457, 1943.
6. Brugsch, T.: Mechanisch bedingter Ikterus, *Med. Klin.* 31: 366, 1935.
7. Davies, D. T., and Dobbs, E. C.: A Study of the Properties of Pure Bilirubin and Its Behavior Toward the van den Bergh Reagent, *Brit. J. Exper. Path.* 8: 316, 1927.
8. Drabkin, D. L., and Austin, J. H.: Spectrophotometric Studies. II. Preparations From Washed Blood Cells; Nitric Oxide Hemoglobin and Sulfhemoglobin, *J. Biol. Chem.* 112: 51, 1935.
9. Ducci, H., and Watson, C. J.: The Quantitative Determination of the Serum Bilirubin With Special Reference to the Prompt-Reacting and the Chloroform-Soluble Types, *J. LAB. & CLIN. MED.* 30: 293, 1945.
10. Eppinger, H.: Die Leberkrankheiten, Berlin, 1937, p. 246, Julius Springer.
11. Fischer, H., and Lindner, F.: Ueber den Umbau des Blutfarbstoffes durch Hefe, *Ztschr. f. Physiol. Chem.* 153: 54, 1926.
12. Hooper, C. W., and Whipple, G. H.: Bile Pigment Metabolism, *Am. J. Physiol.* 40: 332, 1916.
13. Hoppe-Seyler: Thierfelder, Handbuch d. physiol. u. pathol.-chem. Analyse, Berlin, 1924, Julius Springer, 9 Aufl., p. 417.
14. Horsters, H.: Differentialdiagnose des Ikterus nach färberischen Merkmalen, *Ergebn. d. inn. Med. u. Kinderh.* 56: 575, 1939.
15. Kanasaki, K.: The Effect of the Injection of Glucose Upon Green Bile, *Jap. J. Gastroenterol.* 5: 91, 1933.
16. Langhans, T.: Beobachtungen über Resorption der Extravasate und Pigmentbildung in denselben, *Virchows Arch.* 49: 66, 1870.
17. Lemberg, R.: Ueber Dehydrobilirubin, *Liebigs Ann. d. Chem.* 499: 25, 1932.
18. Lemberg, R., and Barcroft, J.: Uteroverdin, the Green Pigment of the Dog's Placenta, *Proc. Roy. Soc., London, s.B* 110: 362, 1932.
19. Lemberg, R.: Bile Pigments. VI. Biliverdin, Uteroverdin, and Oocyan, *Biochem. J.* 28: 978, 1934.
20. Lemberg, R.: Transformation of Haemins Into Bile Pigments, *Biochem. J.* 29: 1322, 1935.
21. Lemberg, R., and Wyndham, R. A.: Reduction of Biliverdin to Bilirubin in Tissues, *Biochem. J.* 30: 1147, 1936.
22. Lemberg, R.: The Disintegration of Haemoglobin in the Animal Body, *Perspectives in Biochemistry*, pp. 137-149, Cambridge University Press, 1937.
23. Lemberg, R., Lockwood, W. H., and Legge, J. W.: Coupled Oxidation of Ascorbic Acid and Hemoglobin. V. Studies on Formation of Bile Pigments From Choleglobin and Verdohaemochromogen and Their Isolation From Erythrocytes, *Biochem. J.* 35: 363, 1941.
24. Malloy, H. T., and Evelyn, K. A.: The Determination of Bilirubin With the Photoelectric Colorimeter, *J. Biol. Chem.* 119: 481, 1937.
25. McNee, J. W.: Experiments on Haemolytic Icterus, *J. Path. & Bact.* 18: 328, 1913.
26. Nisimaru, Y.: Bile Pigment Formation From Hemoglobin, *Am. J. Physiol.* 97: 654, 1931.
27. Rich, A. R.: The Formation of Bile Pigment From Haemoglobin in Tissue Cultures, *Bull. Johns Hopkins Hosp.* 35: 415, 1924.
28. Rich, A. R.: The Formation of Bile Pigment, *Physiol. Rev.* 5: 182, 1925.
29. Schreus, H. T., and Carrié, C.: Untersuchungen zum Gallenfarbstoffwechsel. I. Mitteilung; Abbau des Blutfarbstoffes zu Protoporphyrin durch Leber und andere Organe; nachweisbarer Fermentwirkung, *Klin. Wchnschr.* 13: 1670, 1934.
30. Sumner, J. G., and Nymon, M.: The Oxidation of Bilirubin by Peroxidase, *Science* 102: 209, 1945.
31. Warburg, O., and Negelein, E.: Grünes Haemin aus Blut-Haemin, *Ber. deutsch. chem. Ges.* 63: 1810, 1930.

# DIFFERENTIAL AGGLUTINATION OF HUMAN ERYTHROCYTES

## EVALUATION OF TECHNIQUE

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THE differential agglutination of erythrocytes depends upon a specific reaction between the agglutinin of an antiserum and the agglutininogen of red cells. After transfusion of red cells lacking an agglutininogen present in the cells of the recipient, it is possible to agglutinate the cells of the recipient by use of an appropriate antiserum. Conversely, after transfusion of erythrocytes containing an agglutininogen not present in the cells of the recipient, but for which the recipient's serum has no specific agglutinin, the donated corpuscles can be differentially agglutinated.

Inasmuch as this procedure has many applications in investigative medicine, it was deemed worth while to study the factors affecting the accuracy of the method and to determine the most satisfactory quantitative procedure. The objectives of this paper are (1) to describe the technique currently used by this laboratory and (2) to present the practical, experimental, and statistical considerations which led to the adoption of this technique.

## HISTORICAL

In 1919 Ashby<sup>1</sup> first studied the fate of transfused erythrocytes by giving Group O cells to recipients belonging to one of the other major groups. She found, for example, that after transfusing Group O cells to a Group A recipient, the latter's cells could be agglutinated with anti-A (Group B) serum and that the unagglutinated O cells of the donor could then be enumerated by transferring the serum-cell mixture to a counting chamber. Wiener<sup>2, 3</sup> later demonstrated that differences in MN type could be utilized in a similar manner. After transfusing AN cells, for instance, to an AMN recipient, the latter's cells were agglutinated by anti-M serum and the unagglutinated AN cells of the donor could be counted as in the method of Ashby. By utilizing both ABO and MN differences, it was soon proved feasible to make observations simultaneously on the fate of cells from two or three different donors in the circulation of a single recipient.

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Ashby's technique consisted of filling a leucocyte pipette with capillary blood to the .5 mark. The pipette was then filled to the 1.1 mark with a 20:1 mixture of serum and 4.4 per cent citrate solution. The mixture was expelled into a small test tube, shaken, incubated at 37° C. for forty minutes with shaking every ten minutes, and then placed in an icebox overnight. On the following day the mixture was shaken, a drop placed in a counting chamber, and the cells in 160 small squares in each of two chambers were counted. A tube containing the blood of a nontransfused individual was used as a control to test the agglutinative capacity of the serum, and the same serum was used throughout each experiment. Ashby<sup>4</sup> later advised against refrigeration of serum-cell mixtures because of nonspecific agglutination caused by low temperatures.

In 1922 when Wearn and associates<sup>5</sup> used Ashby's method to determine the life of transfused red cells, they noted that, of the cells which should have been specifically agglutinated, from 20,000 to 50,000 per cubic millimeter remained unagglutinated and they considered 50,000 such cells per cubic millimeter the upper limit for satisfactory sera.

In 1924 Jervell<sup>6</sup> made a comprehensive study of the factors affecting the accuracy of Ashby's technique. He used saline suspensions of cells mixed with agglutinating sera of high titer and found that shaking of serum-cell mixtures for one minute was adequate and that further shaking did not alter the counts.

Landsteiner and co-workers<sup>7</sup> in 1928 first employed M and N factors in a study of the longevity of type M donated cells in a recipient of type N, and Wiener and associates<sup>8, 9</sup> applied M-N differences to the study of transfusion reactions and storage of blood.

Mollison and Young,<sup>10, 11</sup> who applied the principle of differential agglutination to the study of various blood preservatives, drew capillary blood into a hemoglobinometer pipette, mixed it with citrate, and then mixed the cell suspension with antiserum in a flat-bottomed bottle. Anti-A, -B, -M, and -N sera were used, and two individuals enumerated the unagglutinated cells.

Dacie and Mollison<sup>12</sup> in 1943 further refined the technique during the course of their studies on hemolytic anemias. They added 20 c. mm. of capillary blood to 1.0 c.c. of saline, or 0.1 c.c. of venous blood to 5.0 c.c. of saline; the resulting cell suspension was mixed with an equal volume of potent anti-A or anti-B serum, allowed to stand an hour, centrifuged, shaken, and again centrifuged. Anti-M and anti-N sera were used in the same way with the exception that the serum-cell mixtures were allowed to stand two hours and were not placed in the centrifuge.

The technique of Dacie and Mollison was used with notable success by Brown, Hayward and co-workers<sup>13</sup> and by Callender and associates<sup>14</sup> in their investigations of the manner of destruction of transfused normal erythrocytes by normal and anemic recipients. This method also has been employed by Young and Lawrence<sup>15, 16</sup> whose only deviation was that of preparing an initial 1:101 dilution of blood in saline instead of a 1:51 dilution as recommended by Dacie and Mollison.

Other techniques of differential agglutination have been developed and successfully used by Dekkers,<sup>17</sup> Denstedt and co-workers,<sup>18</sup> and Thalhimer.<sup>19</sup> Ebert and Emerson,<sup>20</sup> who made a thorough study of the hemolytic effect of Group O blood and pooled plasma, were the first to describe the use of dried antiserum for differential agglutination, an innovation having advantages which will be enumerated subsequently. It is the technique of these authors<sup>20, 21</sup> that has been followed to the greatest extent in developing the method currently adopted by this laboratory.

In 1924 Isaacs<sup>22</sup> criticized studies employing the technique of differential agglutination of cells. He stated that reticulocytes and nucleated red cells were nonagglutinable and were largely responsible for the error of the method. Subsequent observations in other laboratories and those reported in this paper show clearly, however, that reticulocytes are readily agglutinated by the usual antisera.

#### APPLICATIONS OF THE TECHNIQUE

The applications of the technique of differential agglutination are described in detail in the publications to which references have been made and are reviewed briefly elsewhere.<sup>23</sup> These applications may be enumerated as follows:

1. Determination of the life span of normal red cells by transfusion to normal recipients. By this method the erythrocyte has been found to survive for periods as long as 120 days or more.<sup>14</sup>

2. Study of pathologic destruction of red corpuscles in hemolytic disorders by "cross-determination of the lifetime of erythrocytes."<sup>12, 16, 24, 25</sup> Normal cells are transfused to patients with hemolytic anemia and cells from the latter are transfused to normal recipients in an effort to determine whether intra- or extracorpusecular abnormalities, or both, may be present.

3. Investigation of transfusion reactions in which incompatible donated cells are rapidly destroyed by the action of regular or irregular isoagglutinins of the recipient, or in which the recipient's cells are subjected to the hemolytic effect of transfused plasma or Group O blood.<sup>9, 20, 26, 27</sup>

4. Evaluation of the effects of storage, preservatives, and methods of transport of whole blood and red blood cells.<sup>8, 10, 11, 18</sup>

5. Observations on the selective action of the spleen on abnormal autologous cells as contrasted with normal donated cells.<sup>28, 29</sup>

#### THE TECHNIQUE

Five cubic centimeters of venous blood are withdrawn into a dry syringe with a minimum of stasis, and after removing the needle, the blood is expelled into a bottle containing 4 mg. of dried potassium oxalate and 6 mg. of dried ammonium oxalate.<sup>30</sup> After gentle rotation, 0.5 c.c. of the oxalated blood is transferred with a volumetric pipette to a 100 c.c. volumetric flask which is then filled to the mark with fresh normal saline. After thorough mixing, 0.2 c.c. portions of the 1:200 suspension are transferred with a serologic pipette

to each of four test tubes of approximately 7 mm. inside diameter and 6 cm. length. Tubes 1 and 2 contain only the saline suspension of cells, while tubes 3 and 4 contain 2 to 4 mg. of dried anti-M or anti-A serum.\* This is the amount which can conveniently be held on the broad end of a toothpick, and no more accurate measurement than this appears to be necessary or practical. All the tubes are shaken gently and after about three minutes tubes 3 and 4 containing the serum-cell mixtures are centrifuged for one minute at 1,000 revolutions per minute. They are then shaken for fifteen seconds by rapid flicking against the table and after standing five minutes or longer at room temperature the mixtures are counted.

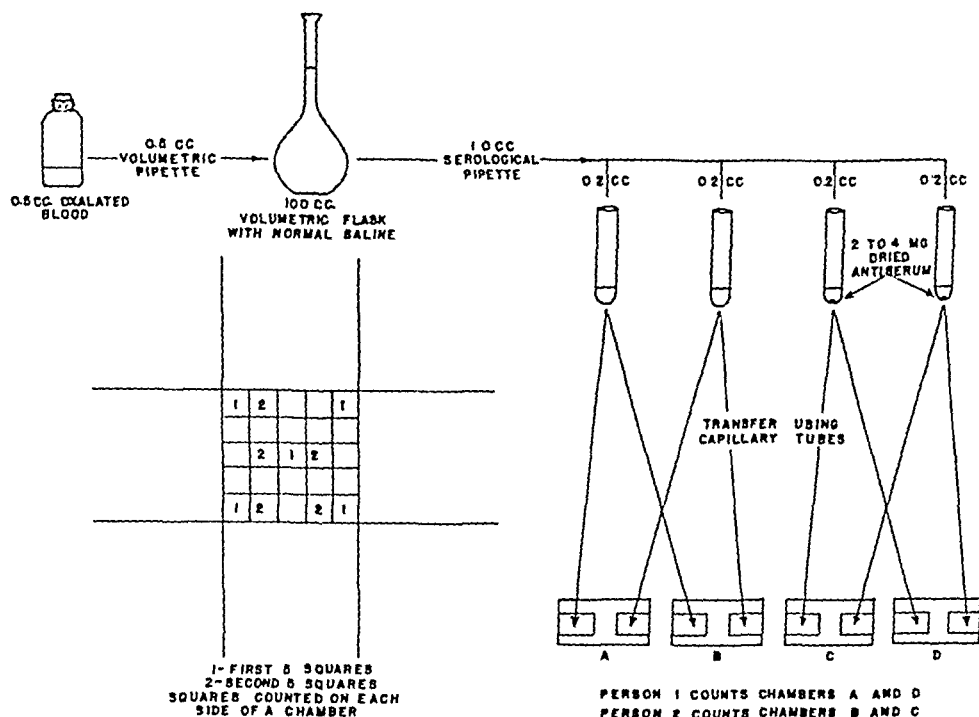


Fig. 1.—Diagram of technique.

After reshaking for fifteen seconds the cell suspensions and serum-cell mixtures are transferred to a bright-line counting chamber by means of large bore (0.8 to 1.2 mm.) capillary tubes which are discarded after a single filling. Each of two individuals fills one side of a chamber from each of the four tubes and counts the red cells in two sets of five squares (each square measuring 200 microns) in each side of a chamber as indicated in Fig. 1. By this procedure, 4 saline and 4 serum counts of five squares each are made by each person, thus giving a total of 8 saline and 8 serum counts. In counting fields where agglutinated cells are present, any square containing a large clump is omitted and an adjacent square is counted in its place. Both persons enumer-

\*The dried antisera used in these studies were purchased from Lederle Laboratories, Pearl River, N. Y.

ate the cells simultaneously and with a minimum of delay. Counts are discarded and repeated if (1) either person suspects a break in technique or (2) the difference between the average counts for five squares is greater than 1.5 standard deviations.

The same lot of serum is used throughout each investigation, if possible, and the serum is tested at the beginning of the experiment, and periodically thereafter, for its ability to agglutinate the cells which possess the specific agglutinin. If less than 10,000 per cubic millimeter of the cells containing the agglutinin for which the antiserum is specific are left unagglutinated, no correction factor is necessary. If from 10,000 to 50,000 cells per cubic millimeter are left unagglutinated, this correction factor is subtracted from each serum count. If more than 50,000 cells per cubic millimeter fail to agglutinate, the serum is considered unsatisfactory for use in this procedure. This means that 99 per cent of the cells must be agglutinated if the suspension used contains 5 million cells per cubic millimeter.

The calculations made after completing each group of counts are most easily presented by citing an example such as that of an OMN recipient transfused with ON erythrocytes. Following such a transfusion, anti-M serum agglutinates nearly all of the recipient's cells and leaves unagglutinated all of the donated cells which are enumerated in the serum count. In this case:

- (1) Total number of red cells per cubic centimeter (that is, OMN + ON cells) = (Average of 8 saline counts of five squares each)  $\times$  10,000
- (2) Number of ON cells per cubic millimeter = (Average of 8 serum counts  $\times$  10,000) - the correction factor
- (3) Number of OMN cells per cubic millimeter = Total number red cells per cubic millimeter - number ON cells per cubic millimeter

If typical figures are substituted in these formulas as examples, the following results are obtained:

- (1)  $425 \times 10,000 = 4,250,000$
- (2)  $(170 \times 10,000) - 20,000 = 1,680,000$
- (3)  $4,250,000 - 1,680,000 = 2,570,000$

It is to be noted that the average of the saline counts made by this method provides an accurate count of the total red cells per cubic millimeter when the figure is multiplied by 10,000.

If the number of unagglutinated cells in the serum-cell mixture is below 100 per set of five squares, it is advisable, because of large Poisson errors, to make a separate saline suspension containing 1, 2, or 4 c.c. of oxalated blood in 100 c.c. of normal saline and to use this suspension for mixing with anti-serum. Counts obtained are then divided by 2, 4, or 8, respectively, in the final calculations.

When liquid antiserum is used instead of the dried preparation, 1.0 c.c. of oxalated blood is ordinarily diluted to 100 c.c. with saline and 0.2 c.c. of the 1:100 suspension is placed in each of four tubes as is done when dried serum is used. To tubes 1 and 2 is added 0.2 c.c. of saline and to tubes 3

and 4 is added 0.2 c.c. of antiserum. The remainder of the procedure is the same with liquid sera, with the exception that it may be necessary to shake the serum-cell mixtures more gently than when dried antisera are used. The optimum amount of shaking may vary from serum to serum, either wet or dry, and it is therefore necessary that this amount be determined for each lot of serum at the beginning of an experiment.

#### FACTORS AFFECTING THE ACCURACY OF THE TECHNIQUE

*Selection of Donor-Recipient Combination Prior to Transfusion.*—Since anti-A, -B, and -M sera give quantitative results, as will be shown in the discussion of antisera, it is possible to use differential agglutination in the combinations given in Table I. Inasmuch as the cells of all individuals in the population contain either the M or the N factor, or both, it is possible to carry out this procedure with all recipients. When Group O blood is given to A, B, or AB recipients, it is advisable to use only the red cells<sup>20</sup> or to add A and B substances to whole blood. The deliberate transfusion of Rh-positive blood into Rh-negative recipients for the purpose of studying differential agglutination is potentially dangerous and unjustifiable, particularly if there are to be repeated transfusions or if pregnancy is anticipated in the case of the Rh-negative female recipient. Likewise, the repeated transfusion of A<sub>1</sub> blood to an A<sub>2</sub> recipient or vice versa may be potentially dangerous, especially if the recipient is suffering from a hemolytic disorder.<sup>21</sup>

TABLE I. MIXTURES OF DONOR'S AND RECIPIENT'S BLOOD MOST SATISFACTORILY USED IN DIFFERENTIAL AGGLUTINATION

RECIPIENT'S GROUP	DONOR'S GROUP	SERUM USED TO DIFFERENTIATE	UNAGGLUTINATED CELLS
A	O	Anti-A	O
B	O	Anti-B	O
AB	O	Anti-A, Anti-B, or both	O
	A	Anti-B	A
	B	Anti-A	B
N	M	Anti-M	N
	MN	Anti-M	N
M	N	Anti-M	N
MN	N	Anti-M	N

*Sampling of Recipient's Blood.*—Best results have been obtained with 5 c.c. samples of venous blood drawn into dry, sterile syringes without stasis and transferred to bottles containing dry oxalate mixture. Capillary blood may be used if venous blood is not readily obtainable, but it is to be kept in mind that some accuracy is sacrificed in taking small samples.\* Capillary blood is most easily drawn with a hemoglobinometer pipette from which a 0.02 c.c. sample is transferred to a tube containing 4.0 c.c. of saline, thus giving a

\*It should be emphasized that the hematocrits of venous and capillary blood may differ significantly and that both may differ widely from the "whole body" hematocrit. The results of differential agglutination tests made after transfusion should be interpreted with these hematocrit variations in mind.

1:200 dilution of cells as in the method employing venous blood. Capillary samples are best taken and diluted in duplicate in order to reduce the error inherent in the use of small volumes.

*Preparation of the Saline Suspension.*—The 0.5 c.c. volumetric pipettes, which are used routinely in transferring venous blood samples to the 100 c.c. flasks, have an error of not more than 0.1 per cent, while  $\pm 16$  c.c. is the tolerance for error of the 100 c.c. volumetric flasks. Multiple dilutions with the accompanying increase in error are avoided by this method. It is important, if accurate results are to be obtained, that saline suspensions be of such density as to provide counts of between 400 and 500 and never less than 200 cells per five squares. Stronger suspensions should be made if the counts do not fall within this range. As previously stated, more concentrated suspensions of cells in saline should be prepared to mix with antiserum if the counts of unagglutinable cells fall below 100 per five squares. The coefficient of variation for a count of 100 is 9 per cent and is larger for counts smaller than 100.

Differential counts are sometimes made on the nonhemolyzed cells remaining in the bottom of each tube used in testing the osmotic or mechanical fragility of mixtures of two types of cells present in peripheral or splenic blood.<sup>28, 29</sup> When this is done, the cells are resuspended in normal saline until the density of the suspension matches that of a standard tube of the same size containing a 1:200 dilution of whole blood having a red cell count of from 4.0 to 5.0 million. Two-tenths cubic centimeter portions of the resuspended cells are then transferred to four small tubes, and the remainder of the procedure is carried out in the usual way. The proportions of the two types of cells can be determined from counts made on such preparations.

#### *The Antiserum.*—

*The Antisera to Be Used:* In light of the present knowledge of the agglutinogens of human erythrocytes, it is theoretically possible to use eleven different antisera for this purpose. These antisera are anti-A, -B, -A<sub>1</sub> (absorbed B), -M, -N, -P, -Rh<sub>0</sub>, -Rh', anti-Rh'', -Hr', and -Hr''. In the final analysis the accuracy of the procedure depends to a great extent on the quality of antiserum used. Of the sera available in our laboratory only anti-M, -A, and -B have been satisfactory.

Dried serum has the advantages of relatively uniform potency, stability, and economy. The fact that dried antiserum, in the amounts used, does not dilute the saline cell suspensions was proven by comparing the counts of saline suspensions of AN cells before and after adding dried anti-M serum in the usual proportions. The difference between the counts was only 2.8 per cent, revealing no apparent dilution or nonspecific agglutination. This experiment was repeated using dried anti-N serum and AM cells and the difference in counts was less than 4 per cent.

*The Proportion of Antiserum and Cell Suspension:* The amount of dried serum, which causes nearly complete specific agglutination and no nonspecific agglutination, has been determined by our experiments to be from 2 to 4 mg. per 0.2 c.c. of cell suspension. Amounts of from 4 to 8 mg. in 0.1 c.c. of cell suspension caused nonspecific agglutination in the case of anti-N serum. For



practical purposes the amount which can be held on the broad end of a toothpick (2 to 4 mg.) gives satisfactory results.

*Correction Factor for Nonagglutinable Cells:* The problem of correction factors based upon counts of unagglutinated cells made prior to transfusion has been extensively dealt with in the literature.<sup>5, 10, 22</sup> To be satisfactory for use in this procedure the serum should leave unagglutinated not more than 50,000 cells per cubic millimeter, or more than 1 per cent of cells containing the specific agglutinin. Frequent retesting of the antisera, particularly liquid antisera, to check the correction factor is advised. As shown in Table II, the nonagglutinable cells amounted to only 0.2 per cent with dried anti-M serum and 0.5 per cent with dried anti-A serum currently in use in this laboratory.

The theory that nonagglutinable cells are reticulocytes or nucleated cells is untenable in view of the work of Callender and associates<sup>14</sup> and the following observations made in this laboratory:

1. The freshly drawn erythrocytes which were left unagglutinated after mixture with anti-A, -B, and -M sera were stained with brilliant cresyl blue and no reticulocytes or morphologically abnormal cells were found.

2. Dried anti-M serum was added to a suspension of MN cells from a patient with chronic hemolytic anemia. Although 51 per cent of the cells were reticulocytes, only 2 per cent were left unagglutinated, which means that most of the reticulocytes were agglutinated as were the mature cells. At the beginning of a previous experiment,<sup>15</sup> when 73 per cent of the erythrocytes from this patient contained reticulum, only 0.5 per cent were left unagglutinated by liquid anti-M serum.

*Potency of Antiserum:* In order to test the effectiveness of various antisera in differential agglutination, suspensions of washed, fresh A<sub>1</sub>MRh+, BNRh+, OMRh-, and A<sub>2</sub>MRh+ cells were prepared in saline. Counts were made on each suspension, 1.0 c.c. portions of each suspension were combined to make a mixture of the four types of cells, and this mixture was then counted. To portions of the mixed cell suspension, anti-A, -B, -A<sub>1</sub>, -M, -N, and -Rh<sub>0</sub> sera were added by the technique described. In order to determine the nonagglutinable cell count, each of these antisera was tested with that suspension of cells containing the agglutinin for which it was specific; for example, the anti-B serum was tested with the BNRh+ cells. Four counts of five squares each were made by each of four individuals on each saline suspension and serum-cell mixture.

This experiment was designed to test the accuracy of differential agglutination with the antisera which were available in our laboratory on a given date. Although the results of this experiment which are shown in Table II apply only to the sera tested, similar observations may be carried out to compare the potency of other sera and to determine the accuracy of the procedure. It is apparent that only the anti-M and anti-A sera tested were sufficiently potent to agglutinate 99 per cent of the cells containing the specific agglutinin. A comparison of expected per cent nonagglutinated cells and the actual per cent of nonagglutinated cells in the mixture of cells is shown in Table II. The

TABLE II. RESULTS OF DIFFERENTIAL AGGLUTINATION OF ARTIFICIAL MIXTURE OF ERYTHROCYTES WITH ANTISERUM

ANTISERA	AVERAGE OF 4 COUNTS BY EACH OF 4 PERSONS	ACTUAL PER CENT NOT AGGLUTINATED*	EXPECTED PER CENT NOT AGGLUTINATED	PER CENT DIFFERENCE + OR -	PER CENT NONAGGLU- TINABLE†
Anti-A (dry)	258	52.6	50.4	2.2‡	0.5
Anti-B (dry)	372	71.4	73.8	2.4§	4.9
Absorbed B (liquid)	206 × 2	41.1	75.6	34.5§	41.7
Anti-M (dry)	122	24.9	26.2	1.3§	0.2
Anti-N (dry)	304	54.1	73.8	19.7§	8.21
Anti-Rh <sub>0</sub> (liquid)	125 × 2	26.0	24.2	1.8‡	26.2

\*Corrected for nonagglutinable cells as shown in last column.

†Counts made on individual cell suspensions.

‡Not agglutinated.

§Agglutinated.

anti-A<sub>1</sub> and anti-N sera yielded results grossly different from those expected. These results illustrate the consequence of using antisera of low potency and specificity. The dried anti-B serum used in this experiment left 4.71 per cent of B cells unagglutinated, whereas previous lots of liquid anti-B serum obtained from donors immunized with A and B substances were considerably more potent. Additional lots of dried anti-B serum have not yet been tested in this laboratory.

Minimum requirements for blood grouping and typing sera have recently been set forth by the National Institute of Health.<sup>32</sup>

#### *The Reaction of Specific Agglutinin and Agglutininogen.—*

*Time:* The time required for maximal agglutination varies widely depending upon the avidity of the serum and whether or not the serum-cell mixture is centrifuged. In our experience and that of other recent investigators,<sup>21</sup> three minutes before centrifuging and five minutes afterward are adequate.

*Centrifugation of Serum-Cell Mixtures:* It is well known that hemagglutination is greatly accelerated by centrifugation of serum-cell mixtures. Dacie and Mollison<sup>12</sup> recommend that the mixtures containing liquid anti-A or anti-B serum be allowed to stand for at least sixty minutes before centrifugation. The experience of this laboratory indicates, however, that a period as short as three minutes is adequate for dried anti-A and anti-M sera and probably for liquid anti-A, anti-B, and anti-M sera. Ebert and Emerson<sup>20</sup> and Emerson and Shen<sup>21</sup> have also found that it is necessary for mixtures containing dried anti-A or anti-B serum to stand only a few minutes before centrifugation.

Since anti-M and anti-N sera are absorbed rabbit sera, nonspecific reactions between these sera and human cells may often be encountered.<sup>3, 19</sup> Dacie and Mollison have cautioned against centrifugation of mixtures containing anti-M or anti-N serum because of the danger of causing nonspecific clumping of type N cells by an occasional lot of liquid anti-M serum, but this has not been true of the lots of dried anti-M serum thus far tested. Both liquid and dried anti-N sera have been noted to cause some nonspecific agglutination in our experience, particularly after centrifugation. If centrifugation is carried out for more than one minute at 1,000 to 1,500 r.p.m. there may be some false agglutination.

*Temperature:* In our experience and that of other workers<sup>2, 10</sup> room temperature is most satisfactory. The titer, and probably the avidity of isoagglutinins is decreased at temperatures as high as 44 to 55° C. which were recommended by Jervell.<sup>6</sup> Refrigeration, on the other hand, as originally used by Ashby<sup>1</sup> may cause nonspecific cold hemagglutination.

*Shaking:* The amount of shaking to which the serum-cell mixture should be subjected is a problem which must be standardized with various sera. We have found that with potent dried antisera vigorous shaking by flicking against the table for fifteen seconds is necessary and if the shaking is too gentle false agglutination may be present. Vigorous shaking may not be necessary, however, when liquid antisera are used. Saline suspensions are shaken in the same fashion as serum-cell mixtures.

*Filling the Chamber.*—For filling the chamber it has been found that disposable glass capillary tubes which are from 0.8 to 1.2 mm. in diameter and 12 cm. in length are convenient and give constant results. When exceptionally large clumps of agglutinated cells are present, it is advisable to avoid them in removing samples from the tubes.<sup>2, 12</sup> When saline cell suspensions were transferred to counting chambers with 1.2 mm. capillary tubes, 0.2 mm. capillary tubes and the stems (0.4 mm.) of Trenner erythrocyte pipettes, the differences in counts were insignificant. In making this comparison each of two persons made four transfers by each of the three methods and counted ten (200 mm.) squares after each transfer. It was found, however, that in transferring serum-cell mixtures irregularities were apt to occur when capillary tubes smaller than 0.5 mm. in diameter were used. Trenner pipettes have not been employed for this purpose because of the time required for their cleaning.

*Counting of Cells.*—Simultaneous counts by two persons of the cells in each tube make it possible to detect the unavoidable errors of technique which occasionally occur. These errors are assumed to exist, even though they have not been observed, when the differences between the counts is greater than 1.5 standard deviations. The counts are then discarded and repeated with different chambers. The 1.5 standard deviation control limit is economical, permitting a loss of only about 12 per cent of "true" counts. The standard deviation of the difference between the counts (that is, the average of four sets of five squares) obtained by two persons is:

$$\sigma (A_1 - A_2) = \sqrt{0.15 (A_1^2 + A_2^2) + .2 (A_1 + A_2)}$$

where  $A_1$  and  $A_2$  are the counts by the two persons (See Statistical Considerations). In the range of counts dealt with, 100 to 500,  $1.5 \sigma$  has the range 20 to 80.

In order to determine the better method a statistical test was done comparing: (1) two persons counting ten squares on each side of the chamber and (2) two persons counting twenty-five squares on only one side of the chamber. By each method 30 saline suspensions of different concentrations were counted. The coefficients of variation for the difference between the average number of cells in five squares (that is, the average counts) found by two persons were corrected for Poisson errors and were found to be (1) 8.2 per cent and (2)

13.4 per cent. Fisher-Z which tests the homogeneity of these values has a probability near 5 per cent. The discrepancy of these values is attributable to chamber error, more thoroughly characterized by Berkson<sup>23</sup> as 4.5 per cent. Since the consistency between counts by different persons is used as a criterion of statistical control, the first method (1) was adopted because of its greater precision.

#### STATISTICAL CONSIDERATIONS

Formulation of the technique presented in this paper was based upon many practical and experimental considerations, some of which required statistical tests and judgments in the management of sampling errors in counts. A count obtained by this technique presumably will be compared with another one under different experimental or clinical conditions. Such comparisons of counts require statistical judgments based on the standard deviations of counts and differences between them. If  $\sigma_A$  is the standard deviation of count A, and  $\sigma_B$  is the standard deviation of count B, the standard deviation of the difference between counts A and B is:

$$\sigma_{(A-B)} = \sqrt{\sigma_A^2 + \sigma_B^2}$$

Similar manipulations of the standard deviation can be found in elementary statistical textbooks for other types of experimental comparisons. A 1.5  $\sigma$  tolerance or error range includes about 88 per cent of random counts. In this laboratory differences between counts beyond the 1.5  $\sigma$  range are conventionally considered nonrandom.

The general formula for the standard deviation of a count by the technique of this paper is given by:

$$\sigma_A = \sqrt{\frac{0.15}{N} \frac{A^2}{M} + \frac{.8A}{MN}}$$

where A is the count, which is the average count of M counts of five squares by each of N persons. The precision of any statistical test using  $\sigma_A$  can be enhanced by increasing M, N, and/or A. A convenient number of counts of five squares is 4 (M = 4) and persons is 2 (N = 2), and counts are held within the range 100 to 500. The first factor (.015) was determined from several hundred counts made under varying circumstances: different values of M, N, and A; saline and different antisera; different recipient-donor combinations, etc.; all of these conditions showed homogeneity of errors. This factor, which should be determined in each laboratory unless the technique described here is followed exactly, represents the relative variation due to random effects in apparatus and technique and limits the precision in high counts. The second error factor (.8A) due to Poisson errors<sup>23</sup> is important in low counts. For A = 100,  $\sigma_A = 9$ . Thus, counts smaller than 100 should be avoided by using cell suspensions of sufficiently low dilution.

Example: Accompanying the numerical example under Technique are the following calculations of errors:

$$(1) \text{ Saline count} = 425 \pm 37$$

$$\text{where } 37 = \sigma = \sqrt{\frac{.015 (425)^2}{2} + \frac{(.8) (425)}{(4) (2)}}$$

$$(2) \text{ Serum count} = 168 \pm 15$$

$$\text{where } 15 = \sigma = \sqrt{\frac{.015 (168)^2}{2} + \frac{(.8) (168)}{(4) (2)}}$$

$$(3) \text{ Saline minus Serum} = 257 \pm 40$$

$$\text{where } 40 = \sigma = \sqrt{(37.)^2 + (15)^2}$$

In similar fashion the difference between (1), (2), or (3) counts for different clinical or experimental conditions can be tested for statistical significance by repeated application of the general formula,  $\sigma_{(A-B)}$ , previously given.

Example: One day after transfusion of ON blood to an OMN recipient, the technique was carried out using dried anti-M serum which agglutinated the recipient's but not the donor's cells. The serum count was 132 and a month later it was 103. The standard deviation for the difference between two counts,  $A = 132$ ,  $B = 103$ :

$$\sigma_{(A-B)} = \sqrt{\frac{.015 (132^2 + 103^2)}{2} + \frac{.8 (132 + 103)}{(4) (2)}} \approx 15$$

#### SUMMARY

1. A technique of differential agglutination of human erythrocytes is presented in detail and its applications in experimental medicine are outlined.
2. The accuracy of the technique has been tested experimentally.
3. The precision of the technique has been analyzed and stated statistically.

#### CONCLUSIONS

1. The technique of differential agglutination gives accurate and precise quantitative results.
2. Antisera of sufficient potency, avidity, and specificity are necessary. Dried antisera (A, B, and M) have the advantages of stability and relatively uniform potency.
3. Reticulocytes are agglutinated in the same manner as mature cells and erythrocytes which fail to agglutinate are not morphologically abnormal.

#### REFERENCES

1. Ashby, W.: The Determination of the Length of Life of Transfused Blood Corpuscles in Man, *J. Exper. Med.* 29: 267, 1919.
2. Wiener, A. S.: Correspondence, *J. A. M. A.* 122: 1779, 1934.
3. Wiener, A. S.: *Blood Groups and Transfusion*, ed. 3, Baltimore, Md., and Springfield, Ill., 1943, Charles C Thomas.
4. Ashby, W.: Some Data on the Range of Life of Transfused Blood Corpuscles in Persons Without Idiopathic Blood Diseases, *M. Clin. North America* 3: 783, 1919.
5. Wearn, J. T., Warren, S., and Ames, O.: The Length of Life of Transfused Erythrocytes in Patients With Primary and Secondary Anemia, *Arch. Int. Med.* 29: 527, 1922.
6. Jervell, F.: Untersuchungen über die Lebensdauer der Transfundierten Roten Blutkörperchen beim Menschen, *Acta path. et microbiol. Scandinav.* 1: 155, 1924.
7. Landsteiner, K., Levine, P., and Janes, M. L.: On the Development of Isoagglutinins Following Transfusions, *Proc. Soc. Exper. Biol. & Med.* 25: 672, 1928.

8. Wiener, A. S., and Schaefer, G.: Limitations in the Use of Preserved Blood for Transfusion, *M. Clin. North America* 24: 705, 1940.
9. Wiener, A. S., and Peters, H. R.: Hemolytic Reactions Following Transfusions of Blood of the Homologous Group With 3 Cases in Which the Same Agglutinin Was Responsible, *Ann. Int. Med.* 13: 2306, 1940.
10. Mollison, P. L., and Young, I. M.: On the Survival of the Transfused Erythrocytes of Stored Blood, *Quart. J. Exper. Physiol.* 30: 313, 1941.
11. Mollison, P. L., and Young, I. M.: In Vivo Survival in the Human Subject of Transfused Erythrocytes After Storage in Various Preservative Solutions, *Quart. J. Exper. Physiol.* 31: 359, 1942.
12. Dacie, J. V., and Mollison, P. L.: Survival of Normal Erythrocytes After Transfusion to Patients With Familial Haemolytic Anaemia (Achloric Jaundice), *Lancet* 1: 550, 1943.
13. Brown, G. M., Hayward, O. C., Powell, E. O., and Wits, L. J.: The Destruction of Transfused Erythrocytes in Anemia, *J. Path. & Bact.* 56: 81, 1944.
14. Callender, S. T., Powell, E. O., and Wits, L. J.: The Life Span of the Red Cell in Man, *J. Path. & Bact.* 57: 129, 1945.
15. Young, L. E., and Lawrence, J. S.: Maturation and Destruction of Transfused Human Reticulocytes; Evaluation of Reticulocyte Experiments for the Measurement of Hemoglobin Metabolism, *J. Clin. Investigation* 24: 554, 1945.
16. Young, L. E., and Lawrence, J. S.: Atypical Hemolytic Anemia; Observations With Particular Reference to the Use of Transfusions in the Study of Hemolytic Mechanisms, *Arch. Int. Med.* 77: 151, 1946.
17. Dekkers, H. J. N.: The Fate of the Transfused Red Blood Cells, *Acta med. Scandinav.* 99: 587, 1939.
18. Denstedt, O. F., Osborne, D. E., Rochlin, I., and Stansfield, H.: The Survival of Preserved Erythrocytes After Transfusion, *Canad. M. A. J.* 48: 477, 1943.
19. Thalheimer, W.: Personal communication.
20. Ebert, R. V., and Emerson, C. P., Jr.: A Clinical Study of Transfusion Reactions: the Hemolytic Effect of Group O Blood and Pooled Plasma Containing Incompatible Isoagglutinins, *J. Clin. Investigation* 25: 627, 1946.
21. Emerson, C. P., and Shen, S. C.: Personal Communications.
22. Isaacs, R.: Properties of Young Erythrocytes in Relation to Agglutination and Their Behavior in Hemorrhage and Transfusion, *Arch. Int. Med.* 33: 193, 1924.
23. Young, L. E.: The Red Blood Cell as an Immunologic Tool, *Indust. Med.* 15: 590, 1946 (abstract). Manuscript in preparation.
24. Singer, K.: Problems of Erythrocyte Disintegration With Particular Reference to the Life Span of the Red Cell, *J. LAB. & CLIN. MED.* 30: 784, 1945.
25. Loutit, J. F.: Discussion on the Life and Death of the Red Blood Corpuscle, *Proc. Roy. Soc. Med.* 39: 757, 1946.
26. Mollison, P. L.: The Investigation of Hemolytic Transfusion Reactions, *Brit. M. J.* 1: 529, 1943.
27. Young, L. E., and Kariher, D. H.: Hemolytic Transfusion Reactions Due to Rh Incompatibility, *J. A. M. A.* 127: 627, 1945.
28. Emerson, C. P., Jr., Shen, S. C., and Castle, W. B.: The Osmotic Fragility of the Red Cells of the Peripheral and Splenic Blood in Patients With Congenital Hemolytic Jaundice Transfused With Normal Red Cells, *J. Clin. Investigation* 25: 922, 1946.
29. Young, L. E., Platzer, R. F., and Lawrence, J. S.: Unpublished Observations.
30. Heller, V. E., and Paul, H.: Changes in Cell Volume Produced by Varying Concentrations of Different Anticoagulants, *J. LAB. & CLIN. MED.* 19: 777, 1934.
31. Young, L. E.: Studies of the Subgroups of Blood Groups A and AB. I. Active and Passive Acquisition of Alpha<sub>1</sub> Agglutinins as a Result of Transfusion, *J. Immunol.* 51: 101, 1945.
32. National Institute of Health: Minimum Requirements for Blood Grouping Serum and Anti-Rh Typing Serum, Dec. 16, 1946.
33. Berkson, J.: Medical Physics. Chicago, 1944. The Year Book Publishers, Inc., p. 110.

## FIBRINOGEN DEFICIENCY: CLINICAL FEATURES AND PROBABLE ETIOLOGIC FACTORS

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**I**N EVALUATING factors which are responsible for a hemorrhagic state, fibrinogen deficiency is seldom considered. Yet, occasionally, as in the case to be described later, it may be an important factor.

Fibrinogen deficiency is rare. It may be either acquired or congenital. Quick<sup>1</sup> described the condition and reviewed the literature. The congenital type is characterized by its hereditary nature, by the tendency to bleed intermittently from birth, frequently oozing from the umbilical stump, and by a low or absent fibrinogen. Nine cases<sup>2</sup> have been reported with no fibrinogen and five<sup>3</sup> with a reduced quantity.

The acquired form has been reported more frequently. Such instances usually show a transient fibrinogen deficiency that has been attributed to an interference in fibrinogen formation. In 1924 Opitz and Silberberg<sup>4</sup> described a fatal case of generalized tuberculosis with hemorrhagic diathesis in a 3-year-old girl with no fibrinogen. At autopsy the liver was replaced by caseous masses and this finding was considered by the authors as evidence that the liver is the source of fibrinogen. In 1927 Knauer<sup>5</sup> reported a case of possible purpura fulminans occurring in a 6-year-old girl during an attack of varicella. Fibrinogen was 0.015 Gm. per 100 c.c. of plasma. No liver damage was evident and it was suggested that the fibrinopenia was due to reticuloendothelial system damage. In 1930 Jürgens and Trautwein<sup>6</sup> found less than 0.1 Gm. per cent fibrinogen in a 52-year-old man with extensive prostatic metastases in the bone marrow. The liver showed subcapsular deposits but otherwise was normal. They suggested that bone marrow has a role in the manufacture of fibrinogen. In 1935 Risak<sup>7, a</sup> examined 230 patients with varied diagnoses and found eighteen with a fibrinogen level below 0.1 Gm. per cent; most of these did not show a bleeding tendency. They included patients with blood dyscrasias, infectious diseases, extensive burns, malignancies, and a miscellaneous group. Waldenstrom<sup>7, b</sup> has recently described three patients with fibrinopenia, hyperglobulinemia, and a tendency to bleed excessively.

In the English literature in 1936 Dieckmann<sup>8</sup> reported three cases of abruptio placentae with a fibrinogen level below 0.1 Gm. per cent and attributed this to the loss of blood. Allibone and Baar<sup>8, b</sup> have described a complete absence of fibrinogen in an infant with obliteration of the bile ducts and biliary cirrhosis of the liver. The fibrinogen was restored to normal with treatment; however, progressive liver damage continued and death occurred in three months. They reviewed the literature, found fibrinogen normal in cases of severe liver damage, and concluded that a decrease in fibrinogen may or may not occur when the liver is diseased and that the site of formation in the liver

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and bone marrow is fairly well established. Recently in 1946 Field and Dam<sup>9</sup> reviewed the factors in production of fibrinogen and concluded that in the chick the fibrinogen levels are influenced by dietary regimen. Apparently an inadequate protein intake produced elevated fibrinogen levels which were attributed to a metabolic disturbance of the liver.

The fibrinogen level at which a hemorrhagic state appears is controversial. In 1931 Peters and Van Slyke<sup>10</sup> gave the normal range for plasma fibrinogen as 0.2 to 0.4 Gm. per cent. In 1930 Jürgens and Trautwein<sup>6</sup> considered 0.15 to 0.12 Gm. per cent as the critical level below which blood did not coagulate. Risak<sup>7, a</sup> cited a patient with constitutional fibrinogenopenia whose son had only 0.02 Gm. per cent fibrinogen and no bleeding tendencies. In many cases the bleeding is intermittent even though the fibrinogen remains low or even absent. This inconsistency has been attributed to the existence of additional factors. Rabe and Salomon<sup>2, a</sup> thought some change in vascular permeability may be necessary. Several authors<sup>2, b, d, e, f, b</sup> have commented on the association of a thrombopenia and decreased fibrinogen. Allibone and Baar<sup>3, b</sup> reported a case of congenital fibrinogenopenia with hemorrhagic manifestations with a value of 0.11 Gm. per cent and reviewed the theory that a deficiency in the factor of the coagulation mechanism may be compensated by the remaining factors. Henderson and associates<sup>2, b</sup> concluded that the threshold is about 0.06 Gm. per cent.

Because the number of cases so far reported is small, a recent case encountered by us is described in detail.

#### CASE HISTORY

O. W.,\* a 51-year-old farmer's wife, was first admitted to Wesley Memorial Hospital on Aug. 8, 1944, with complaints of diarrhea and generalized weakness both of several months duration. No evidence of a hemorrhagic tendency was present at that time, and the diagnosis was multiple dietary deficiency resembling sprue. The patient was discharged on a high caloric, high protein, high vitamin diet plus supplemental vitamins. On this therapy the diarrhea subsided in about a month and she felt greatly improved, so much so that in May, 1945, nine months after the first admission, she stopped the specific diet and the vitamin supplements. However, the food intake remained abundant and varied. Late in June, 1945, the diarrhea recurred and the weight dropped from 116 to 100 pounds. In August, ten days prior to the second entry, large black and blue areas appeared on the skin. A few days later the patient's knees became painful and discolored. Two small skin abrasions oozed constantly. On the evening before admission she injured her gum with a pin, producing bleeding which continued until about thirty-six hours after entry.

At the time of admission the patient was emaciated. Ecchymotic areas covered all extremities and were numerous about the head. Abrasions on the arm were oozing slightly. The gum at the site of the injury was bleeding freely. The knees were swollen and tender. The skin of the hands, legs, and feet was unusually firm and thick. The blood pressure was 118/80. No abnormalities of the heart, lungs, and abdomen were noted on physical examination. Throughout the next two days bleeding into the tissues, from the gum and urinary tract, became so severe that termination of the hemorrhagic state was urgently needed.

Factors involved in the coagulation mechanism† were considered. As noted in Table I, the coagulation time was seventy minutes. Since calcium is almost invariably present in sufficient concentration to promote thrombin formation when the other factors are present, a possible calcium deficiency seemed unlikely as the cause. A low ascorbic acid level could

\*Patient seen through the courtesy of Dr. Edgar C. Cook of Mendota, Ill.

†Prothrombin plus calcium plus thromboplastin equals thrombin; fibrinogen plus thrombin equals fibrin.



have accounted for increased capillary fragility but not for a prolonged coagulation time. The platelets were only slightly reduced in number (although they may have been functionally abnormal). Consequently, at first the defect was thought to be a decreased prothrombin concentration. Ten milligrams of vitamin K were administered intravenously at eight-hour intervals. A blood transfusion was given three hours after the first dose of vitamin K. The transfusion was followed by immediate cessation of bleeding. It is impossible to state the degree to which the vitamin K, on one hand, and the blood transfusion, on the other, contributed to the termination of bleeding.

This patient has returned for observation on two occasions. There has been no further bleeding, the weight remained unchanged, but she still has irregular periods of diarrhea. Firmness and thickness of the skin of the hands and forearms have persisted.

The significant laboratory findings are tabulated in Tables I to IV. A fibrinogen level of 0.04 Gm. per cent was reported; this value is well below the critical level indicated by most authors. Consequently, it seemed probable that fibrinogen deficiency was an important factor in the bleeding tendency of this patient. During the acute bleeding episode hematuria was noted. Small amounts of free hydrochloric acid were present in the gastric contents on each admission. Stool analysis and proctoscopy showed no abnormalities. The total fat content of dried feces was 22 per cent. The bone marrow showed no significant change; erythropoiesis was normoblastic.

A deficiency in both fibrinogen and prothrombin probably existed simultaneously in this patient. Estimation of either substance depends on the presence of the other essentials of the clotting mechanism. Spontaneous clot formation did occur in the calcified plasma used for fibrinogen determination, and sufficient repetition of analyses by two different methods in separate laboratories verified the exceedingly low level of fibrinogen in the blood. A comparison of the results obtained by the addition of thrombin and by allowing spontaneous coagulation to occur after recalcification of the plasma indicate that a slight difference is obtained by the two methods on either fibrinopenic or normal blood. Spontaneous coagulation was delayed for hours in the most fibrinopenic blood, and the clot formed at the lowest fibrinogen levels reported did not possess the physical properties ordinarily observed in freshly coagulated fibrin. In our experience the most satisfactory method of fibrin estimation is the direct determination of nitrogen in the coagulum after it has been collected and washed. The addition of calcium to plasma results in a somewhat higher value for fibrin as compared to that obtained when thrombin alone is added. This difference, although representing only 0.02 to 0.04 Gm. per cent fibrin, was quite consistently obtained on different samples of blood. Since the determination of prothrombin assumes a normal fibrinogen concentration, prothrombin estimation here would be in error. In only a few of the previous cases of fibrinopenia reported by other authors has prothrombin been considered. Allibone and Baar<sup>3, 6</sup> reported no clot in their prothrombin estimations and discussed the relationship between prothrombin and other coagulation factors. They also pointed out that the routine

TABLE I. COAGULATION FACTORS

	8/8/44	8/18/45	8/22/45	8/24/45	8/24/45	8/27/45	11/9/45	2/11/46
Fibrinogen <sup>13</sup> (grams per cent)		0.04*	0.06	0.078*	0.18	0.18	0.24	0.30
Coagulation time in minutes (Lee-White)		70	6	10			5	5
Prothrombin time in seconds <sup>14, 15</sup>								
Patient		No clot	22	70			30	32
Normal		25	23	35			27	26
Bleeding time in minutes (Duke)		3½						
Clot retraction (twenty-four hours)		Slight						Normal
Platelet count (cubic millimeters)		220,000	140,000			146,000	205,000	161,000
Bumpel-Leede				Positive				
Ascorbic acid		0.16	0.26			1.32	0.88	1.22

\*Blood transfusions were given August 19 and 24.

estimation of prothrombin assumes a normal fibrinogen. Quick<sup>1</sup> has stated that the bleeding in acute yellow atrophy frequently attributed to low fibrinogen is very likely due to severe hypoprothrombinemia.

Further studies were made in an effort to determine the cause of the fibrinogen and prothrombin deficiencies. Liver function was only slightly impaired as judged by the tests employed (Table II). The changes in liver function which were observed are such as one might expect to find in chronic impairment of intestinal absorption. The chronic diarrhea, gross evidence of malnutrition, flat oral glucose tolerance curve (as compared to the normal intravenous curve, Fig. 1), and low plasma vitamin A and carotene levels are all evidence of impaired gastrointestinal absorption. The moderate macrocytic anemia (Table III) and x-ray findings of dilated loops of intestine (Fig. 2) are other manifestations compatible with the diagnosis of a nutritional deficiency which would necessarily be due to impaired absorption since the patient had been on an adequate diet.

The change in intestinal absorption may have been due to a "generalized scleroderma"<sup>11</sup> involving the intestine as well as the skin. However, a skin biopsy showed no abnormality other than edema. The patient's physician recently reported that the skin of the lower legs and feet has become markedly thicker during the months after she left the hospital.

Sprue was considered as a diagnosis. However, at no time did the patient complain of glossitis, and free acid was always found in the gastric contents. Nevertheless, the low plasma vitamin A and carotene levels and the presence of macrocytosis suggest sprue.

Intestinal absorption may have been affected by the pancreatic insufficiency as indicated by the poor amylase output to intravenous secretin (Table IV). The lack of therapeutic response to oral pancreatin, however, argues against pancreatic function as other than a contributory factor to the picture. The findings of fibrinogenopenia in the absence of marked

TABLE II. STUDIES OF HEPATIC FUNCTION

	8/8/44	8/20/45	8/27/45	11/7/45	2/11/46
Bromsulphalein (thirty minutes)			Negative		
I.V. hippuric acid test (0.7 to 0.95 Gm.)			0.3595	0.3469	0.4539
Cephalin flocculation					Negative
Total plasma protein (grams per cent)	4.68	6.18	5.72	5.30	6.03
Albumin	1.2		3.04	3.3	3.81
Globulin	3.48		2.68	2.0	2.04
Icterus index				6.5	

TABLE III. COMPOSITION OF BLOOD

	8/8/44	8/17/45	8/21/45	8/27/45	11/6/45	2/11/46
R.B.C. (million per cubic millimeter)	3.15	4.0	3.92	4.29	3.67	3.59
Hemoglobin (grams)	10	13.0		13.0	12.5	12.0
W.B.C. per cubic millimeter	3,450	6,050		6,250	5,000	4,750
Macrocytosis		Present			Present	Present
Hematocrit per cent					45	41
Urea nitrogen (milligrams per cent)	14.1			10.3		
Serum calcium					10.5	8.1
Pyruvic acid (0.55 to 0.95 mg.)		0.8				0.8
Carotene (100 to 350 I.U.)		13				0
Vitamin A (100 to 250 I.U.)		47				60

TABLE IV. PANCREATIC AMYLASE IN PATIENT AND NORMAL ADULT

SAMPLE	DUODENAL FLUID		AMYLASE		NORMAL RESPONSE OUTPUT
	TOTAL	C.C./MIN.	CONCENTRATION	OUTPUT	
Before secretin*	10	0.5	0	0	30,000
Ten minutes after	28	2.8	600	16,800	77,000
Twenty minutes after	5	0.5	690	3,470	16,300
Thirty minutes after	6	0.6	840	5,050	17,400

\*Response to intravenous secretin expressed as milligrams of maltose liberated per cubic centimeter of pancreatic juice.<sup>12</sup> We are indebted to Dr. H. G. Greengard for the secretin used in this test and for the amylase determinations.

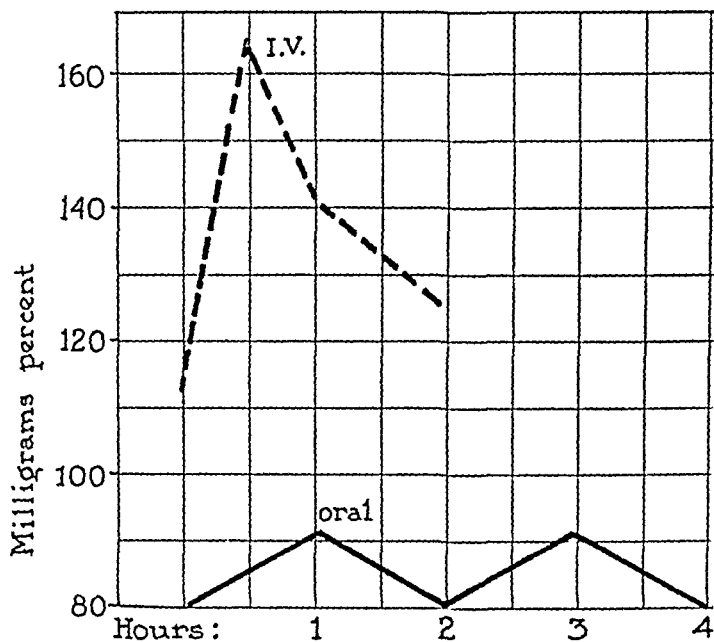


Fig 1—Comparison of oral and intravenous glucose tolerance curves



Fig 2—Persistence of barium in the small bowel and duodenum five hours after ingestion

hepatic insufficiency coupled with impaired intestinal absorption suggests that there may be some nutritional factor that is essential for the formation of fibrinogen by the body.

Treatment has consisted of supplemental and replacement measures. No further bleeding has been reported.

#### SUMMARY

A review of the literature reveals a paucity of reports in which fibrinogenopenia was proved to be an important factor in the production of a severe hemorrhagic state. It is for this reason that this instance is reported in detail. Whether the low fibrinogen level was due to the inability of the liver to synthesize this protein or to impaired absorption of its precursors from the gastrointestinal tract is not known. The evidence points to the latter cause. The cause of poor absorption in this case may be sclerosis of the intestinal wall similar to the changes recognized as scleroderma in the skin.

#### REFERENCES

1. Quick, A. J.: *The Hemorrhagic Diseases and the Physiology of Hemostasis*, Springfield, Ill., 1942, Charles C Thomas, Publisher, pp. 184-191.
2. (a) Rabe, F., and Salomon, E.: Ueber Faserstoff mangel im Blute bei einem Falle von Hämophilie, *Deutsches Arch. f. klin. Med.* 132: 240, 1920.  
(b) Opitz, H., and Frei, M.: Ueber eine neue Form der Pseudohämophilie, *Jahrb. f. Kinderh.* 94: 374, 1921.  
(c) Wolff, J.: Verblutungstod wegen Fibrinmangels, *Jahrb. f. Kinderh.* 148: 33, 1936.  
(d) Macfarlane, R. G.: A Boy With No Fibrinogen, *Lancet* 1: 309, 1938.  
(e) Schönholzer, G.: Die hereditäre Fibrinogenopenie, *Deutsches Arch. f. klin. Med.* 184: 496, 1939.  
(f) Glanzmann, E., Steiner, H., and Keller, H.: Konstitutionelle angeborene Afibrinogenämie und Fibrinopenie im Kindesalter, *Schweiz. Med. Wchnschr.* 70: 1243, 1940.  
(g) Castex, M. R., Pavlovsky, A., and Bonduel, A.: Fibrinopenia congénita, *Medicina*, Buenos Aires 4: 46, 1943.  
(h) Henderson, J. L., Donaldson, G. M. M., and Scarborough, H.: Congenital Afibrinogenemia; Report of a Case With Review of the Literature, *Quart. J. Med.* 14: 101, 1945.
3. (a) Risak, E.: Konstitution und haemorrhagische Diathesen, *Wien. klin. Wchnschr.* 47: 1192, 1934.  
(b) Allibone, E. C., and Baar, H. S.: Fibrinogen Deficiency as a Factor in Haemorrhagic Disease, *Arch. Dis. Childhood* 18: 146, 1943.
4. Opitz, H., and Silberberg, M.: Afibrinogenämie und Thrombopenie infolge aus gedehnter hepato-lienaler Tuberkulose, *Klin. Wchnschr.* 3: 1443, 1924.
5. Knauer, H.: Das Krankheitsbild der Purpura fulminans, *Jahrb. f. Kinderh.* 118: 1, 1928.
6. Jürgens, R., and Trautwein, H.: Ueber Fibrinopenie (Fibrinogenopenie) beim Erwachsenen, nebst Bemerkungen über die Herkunft des Fibrinogen, *Deutsches Arch. f. klin. Med.* 169: 28, 1930.
7. (a) Risak, E.: Die Fibrinopenie, *Ztschr. f. klin. Med.* 128: 605, 1935.  
(b) Waldenstrom, J.: Incipient Myelomatosis or "Essential" Hyperglobulinemia With Fibrinogenopenia—a New Syndrome? *Acta med. Scandinav.* 117: 216, 1944.
8. Dieckmann, W. J.: Blood Chemistry and Renal Function in Abruptio Placentae, *Am. J. Obst. & Gynec.* 31: 734, 1936.
9. Field, J. B., and Dam, H.: Influence of Diet on Plasma Fibrinogen in the Chick, *J. Nutrition* 31: 509, 1946.
10. Peters, J. P., and Van Slyke, D. D.: *Quantitative Clinical Chemistry*, Baltimore, 1931, Williams & Wilkins Company.
11. (a) Goetz, R. H.: The Pathology of Progressive Systemic Sclerosis (Generalized Scleroderma) With Special Reference to Changes in Viscera, *Clin. Proc.* 4: 337, 1945.  
(b) Pugh, D. G., Kræle, W. F., and Margulies, H.: Scleroderma With Involvement of the Viscera; Report of a Case, *Proc. Staff Meet., Mayo Clin.* 20: 410, 1945.
12. Schmidt, C. R., Greengard, H., and Ivy, A. C.: Comparison of Methods for Quantitative Estimation of Diastase in Duodenal Fluid, *Am. J. Digest. Dis.* 1: 618, 1934.
13. Hawk, P. B., and Bergeim, O.: *Practical Physiological Chemistry*, ed. 11, Philadelphia, The Blakiston Company, pp. 453.
14. Kato, K.: Microprothrombin Test With Capillary Whole Blood, *Am. J. Clin. Path.* 10: 147, 1940.
15. Ziffren, S. L., Owen, C. A., Hoffman, G. R., and Smith, H. P.: Simple Bedside Test for Control of Vitamin K Therapy, *Am. J. Clin. Path., Tech. Supp.* 4: 13, 1940.

## BIOCHEMICAL STUDIES DURING MALARIAL AND ARTIFICIAL FEVERS

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THERE have been numerous inquiries into the metabolic alterations produced by fever.<sup>1-10</sup> Recent communications, however, reveal a diversity of opinion<sup>9, 11, 12</sup> and a surprising paucity of observations made during the actual course of a febrile episode. Despite the current universal interest in malaria and the number of investigations directed at this disease, there are few precise data available regarding variations produced in the chemical constituents of the blood.

We were accidentally led into the study of this subject as the result of an observation on a patient who developed tetany during the course of an attack of recurrent malaria. There was present in this case neither hypocalcemia nor alkalosis but instead a profound degree of hypophosphatemia (serum inorganic phosphorus, 0.9 mg. per cent). Since this depletion of phosphorus was found to be transitory, the serum phosphorus having returned to 3.2 mg. per cent within twenty-four hours, it was thought that investigation of other patients with malaria might reveal similar findings. Accordingly, serum phosphorus estimations were made in eighteen additional cases during or within two hours after a chill. None of these showed evidence of tetany. Among these, fourteen patients exhibited serum phosphorus levels between 1.5 and 3.0 mg. per cent. In the remaining four the phosphorus level was normal. It was noted, however, that in those instances in which the serum phosphorus had been normal or at the low borderline level (3.0 mg. per cent) blood samples had been procured rather early in the chill.

Although hypophosphatemia had been described previously in association with the malarial paroxysm,<sup>9</sup> it appeared that no accurate relationship had been established for its time of occurrence or duration. Moreover, there was no conformance in opinions<sup>11-13</sup> regarding the changes effected in other blood constituents. It was decided, therefore, to carry out a study under controlled circumstances and further to determine if possible the existence of parallel changes in nonmalarial fever.

### MATERIALS AND METHODS

The study was carried out on three groups of patients as follows:

*Group A.*—Serum phosphorus levels were determined at serial intervals during sixteen febrile paroxysms in twelve patients with *Plasmodium vivax* malaria. The time of onset of the chill was noted and its relationship to the procurement of the various blood samples established (Table I).

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TABLE I. SERUM INORGANIC PHOSPHORUS IN RELATION TO ONSET OF MALARIAL PAROXYSM

PATIENT	SERUM PHOS- PHORUS AT CHILL ONSET (MG. %)	SERUM PHOS- PHORUS 1 HR. AFTER CHILL (MG. %)	SERUM PHOS- PHORUS 2 HR. AFTER CHILL (MG. %)	SERUM PHOS- PHORUS 3 HR. AFTER CHILL (MG. %)	SERUM PHOS- PHORUS 4 HR. AFTER CHILL (MG. %)	SERUM PHOS- PHORUS 5 TO 8 HR. AFTER CHILL (MG. %)	SERUM PHOS- PHORUS 24 TO 72 HR. AFTER CHILL (MG. %)
W. J. H. (a)*	---	4.1	---	1.3	1.2	1.6	3.6
W. J. H. (b)*	3.2	2.7	---	---	---	---	---
L. F.	---	3.2	2.8	3.0	---	---	4.1
J. G. B.	---	2.4	1.2	---	2.0	3.5	3.9
J. P. W.	---	1.3	2.4	2.8	3.1	---	3.1
W. S.	2.9	2.2	2.9	3.0	3.4	1.8	3.0
R. G. S.	3.2	---	1.7	---	---	---	3.6
R. E. S. (a)	3.0	---	---	2.5	---	3.6	---
R. E. S. (b)	---	2.3	---	---	---	2.9	3.0
R. W. S.	4.2	---	2.0	---	---	---	3.7
H. D. P.	---	---	3.0	3.2	3.3	3.4	---
A. M. (a)	---	---	1.8	2.4	2.0	---	---
A. M. (b)	2.5	---	1.4	1.0	---	---	---
T. L. J. (a)	---	---	3.1	2.1	2.1	---	3.0
T. L. J. (b)	---	2.8	2.2	1.6	---	---	---
P. H.	3.4	2.3	2.0	1.8	---	---	4.2

\* (a) and (b) refer to two successive paroxysms in the same patient without therapy.

*Group B.*—Fifteen patients with benign tertian malaria were selected with a view to excluding complicating diseases or injuries which might in some way influence observations. The studies were instituted within a relatively short period after the onset of the chill and were carried out serially until the temperature fell. Therapy or food were withheld throughout the period of observation, the patients receiving neither antimalarial nor antipyretic drugs. Water was permitted as desired. Records were kept of the temperature changes and their relationships to the time of collection. Both clotted and nonclotted samples were obtained. The anticoagulant employed in the latter consisted of sodium fluoride and mercuric chloride designed to inhibit glycolysis. Spectrophotometric analyses of the following substances were performed: serum inorganic phosphorus,<sup>14, 15</sup> serum calcium,<sup>16</sup> serum magnesium,<sup>17</sup> serum potassium,<sup>18, 19</sup> blood sugar,<sup>20, 21</sup> and total blood-reducing substances.<sup>22</sup> In addition to the afore-mentioned tests, serum protein determinations<sup>23</sup> also were carried out.

The results of these analyses are shown in Table II. Two additional patients (Cases XVI and XVII) were subsequently studied to determine if there was any relationship between the hypophosphatemia and the blood creatine and creatinine (Table III). Observations were carried out under the same conditions as previously outlined.

*Group C.*—This group consisted of ten patients subjected to artificial fever therapy. Seven of these with minor eye ailments were treated in a fever cabinet in which the temperature was raised to 104° F. (rectal) and maintained for one and one-half hours. The other three patients with paresis, were treated in a similar apparatus in which body temperature was raised to 106° F. (rectal) and maintained at this level for four hours. Fasting blood samples were obtained before the patients entered the fever cabinet and temperatures were recorded continuously by means of a thermocouple thermometer. Blood samples were obtained at intervals during the rise and fall of temperature as in Group B. The seven patients with eye disorders received only a liter of water per os during the course of the treatment and no preliminary medication. They were in a fasting state. The three patients with paresis also were in a fasting state, but they were given 200 mg. of ascorbic acid by mouth before entering the cabinet and while febrile were permitted a liter of 2 per cent saline solution.\* The results in this group are summarized in Table IV.

\*This was the routine at the Norristown State Hospital.



TABLE II—CONT'D

CASE	TIME* (HR.: MIN.)	TEMPER- ATURE (° F.)	SERUM PO <sub>4</sub> (MG. %)	SERUM Ca (MG. %)	SERUM Mg (MG. %)	SERUM K (MG. %)	TOTAL PROTEIN (%)	BLOOD GLUCOSE (MG. %)	REDUCING
									SUB- STANCE† (MG. %)
IX	0:20	99.6	2.7	10.9	3.1	--	--	130	12
	1:30	103.0	1.5	11.2	4.5	--	--	126	11
	2:30	104.0	0.8	11.0	2.5	21	--	170	6
	3:30	103.6	1.1	11.2	3.2	19	--	152	3
	4:30	102.0	1.6	11.1	2.4	21	--	134	4
	5:30	101.4	2.4	11.3	2.7	17	--	156	7
	8:00	99.4	4.3	11.3	3.9	18	--	133	10
X	0:10	99.0	4.5	9.3	--	--	6.8	98	--
	1:00	102.6	2.8	10.3	--	--	6.5	110	--
	2:00	105.6	2.2	9.7	--	--	6.0	138	--
	3:00	105.6	1.8	10.5	--	--	6.0	142	--
	4:00	105.2	3.9	10.4	--	--	6.0	158	--
	6:30	97.6	4.2	10.5	--	--	6.2	179	--
	10:30	97.6	3.6	10.6	--	--	6.9	184	--
XI	1:00	103.0	2.5	10.1	2.1	19	6.8	132	--
	1:15	105.0	2.1	10.3	1.8	18	6.5	136	--
	1:40	104.6	1.9	10.3	3.6	18	6.2	146	--
	2:40	105.0	1.4	10.3	2.3	17	5.8	146	--
	3:00	104.0	1.1	10.3	1.9	19	5.8	144	--
	4:25	103.2	1.6	10.5	2.4	16	5.8	149	--
	5:30	101.4	3.3	10.5	1.9	24	6.2	140	12
	13:15	99.6	4.3	10.3	1.9	26	6.2	124	13
	20:30	98.0	3.7	10.3	1.8	18	6.2	113	13
XII	0:10	97.0	5.3	11.1	5.0	21	--	118	13
	0:45	104.2	3.9	10.9	2.7	22	--	139	10
	1:30	103.0	2.8	10.7	3.2	21	--	160	7
	2:15	102.0	1.9	10.8	2.1	22	--	141	7
	6:05	101.0	3.9	11.2	2.4	22	--	130	8
	6:30	100.0	4.4	11.2	4.0	18	--	136	9
	8:45	99.0	4.8	11.0	3.5	18	--	138	12
	11:45	98.0	5.1	11.1	2.1	18	--	120	7
XIII	1:50	99.6	1.2	10.6	3.0	17	5.9	152	8
	3:00	103.4	3.3	10.7	1.4	16	5.9	130	4
	4:00	103.0	--	10.7	--	--	--	146	--
	4:45	102.4	3.1	10.8	1.6	19	5.9	176	4
	6:50	99.4	4.2	10.8	1.6	18	5.9	196	2
	17:15	98.2	3.8	10.8	2.0	20	5.7	126	2
XIV	0:30	101.8	2.1	10.5	2.2	18	6.0	168	20
	2:00	104.0	1.5	10.5	2.3	15	5.8	190	17
	3:00	104.6	1.1	10.7	3.7	15	5.5	170	12
	4:00	105.2	0.9	10.4	5.5	--	5.1	170	14
	7:00	100.2	3.4	11.1	3.9	22	5.5	220	18
	20:00	98.2	4.1	10.5	5.3	30	7.2	220	14
XV	0:20	104.4	2.0	9.8	1.6	19	6.2	140	6
	1:30	105.2	1.7	10.8	3.0	16	5.8	220	6
	2:40	104.6	1.3	10.6	2.0	17	5.9	250	10
	4:30	103.8	1.9	10.9	2.7	16	5.9	236	8
	5:45	103.2	2.9	10.9	1.3	19	5.9	196	2
	7:55	99.6	4.1	10.8	1.4	17	5.9	212	14
	9:10	99.0	4.9	10.8	3.0	21	5.9	168	5
	11:30	98.6	4.9	10.5	1.7	21	5.9	168	6

\*Interval following the onset of the chill.

†Total reducing substances in blood other than glucose; results expressed in terms of milligrams per cent of glucose.



TABLE III. BLOOD CREATINE AND CREATININE STUDIES DURING MALARIAL PAROXYSMS

CASE	TIME* (HR.:MIN.)	TEMPERA- TURE (° F.)	SERUM PO <sub>4</sub> (MG. %)	BLOOD GLUCOSE (MG. %)	BLOOD CREATININE (MG. %)	BLOOD CREATINE (MG. %)	SERUM PROTEIN (%)
XVI	0:10	99	2.5	106	1.5	5.7	5.8
	0:30	103.8	2.3	106	1.6	6.0	5.5
	0:45	105.6	2.1	126	1.5	5.2	6.0
	1:30	105.0	2.5	154	1.5	5.7	5.5
	2:30	105.3	1.3	148	1.5	5.5	5.8
	3:40	105.0	0.8	154	1.5	5.0	5.5
	5:40	104.3	1.0	154	1.6	4.9	5.8
	6:40	102.3	1.9	154	1.7	5.0	6.2
	7:40	99.8	3.6	154	1.8	5.3	5.5
	23:00	97.0	3.8	122	1.6	5.3	6.2
XVII	0:10	99	2.6	122	1.4	6.6	6.5
	0:50	103.6	2.4	144	1.5	5.7	6.5
	1:20	104.6	1.9	162	1.3	6.3	5.8
	2:50	104	1.7	178	1.4	6.6	6.0
	5:50	103	1.9	132	1.4	5.8	6.0
	7:20	102.2	2.9	90	1.4	5.8	6.5
	9:30	104.0	3.0	136	1.4	5.8	6.5
	12:00	101.2	3.6	150	1.5	6.1	6.5

\*Interval following the onset of the chill.

TABLE IV. BIOCHEMICAL STUDIES IN PATIENTS WITH ARTIFICIALLY INDUCED FEVER

CASE	TIME* (HR.: MIN.)	TEMPER- ATURE (° F.)	SERUM PO <sub>4</sub> (MG. %)	SERUM Ca (MG. %)	SERUM Mg (MG. %)	SERUM K (MG. %)	TOTAL PROTEIN (%)	BLOOD GLUCOSE (MG. %)	REDUCING SUB- STANCE† (MG. %)
I	0:00	98.6	4.5	9.8	--	--	7.3	92	--
	1:00	102.0	4.1	9.6	--	--	7.1	116	--
	1:15	104.0	3.9	9.8	--	--	6.8	126	--
	2:35	104.0	3.3	9.6	--	--	6.7	128	--
	3:30	102.0	2.8	9.6	--	--	6.7	140	--
	3:45	99.0	3.2	9.2	--	--	7.1	163	--
II	0:00	97.6	2.1	10.4	--	--	7.2	118	--
	1:10	102.0	1.5	10.5	--	--	7.4	106	--
	1:45	103.2	1.1	11.0	--	--	7.8	130	--
	3:00	103.2	0.7	11.7	--	--	7.6	134	--
	3:45	99.0	0.9	10.6	--	--	7.2	134	--
III	0:00	99.4	3.5	10.9	--	--	6.5	109	--
	0:40	102.0	4.1	10.9	--	--	6.5	106	--
	1:35	104.0	3.8	10.9	--	--	6.5	108	--
	3:00	104.0	3.7	10.8	--	--	6.7	108	--
	3:20	99.2	3.2	10.9	--	--	6.5	102	--
IV	0:00	97.0	3.7	10.9	--	21	6.6	175	--
	1:00	102.0	3.1	10.9	--	20	6.6	170	--
	1:50	104.0	2.5	10.9	--	18	6.6	188	--
	3:15	104.2	2.1	10.9	--	18	7.1	197	--
	3:35	102.0	2.2	11.0	--	17	6.5	294	--
	3:45	98.8	2.6	11.0	--	18	6.5	276	--
V	0:00	98.2	4.1	10.8	1.4	21	6.3	120	--
	0:40	102.0	3.5	11.0	1.6	24	6.5	132	--
	1:10	105.0	2.6	11.5	2.2	24	6.3	136	--
	3:00	104.7	1.7	11.5	1.8	21	6.6	205	--
	3:15	102.0	1.9	11.1	1.2	19	6.3	188	--
	3:30	99.0	2.2	11.1	1.3	20	6.3	176	--

TABLE IV—CONT'D

CASE	TIME* (HR.: MIN.)	TEMPER- ATURE (° F.)	SERUM PO <sub>4</sub> (MG. %)	SERUM Ca (MG. %)	SERUM Mg (MG. %)	SERUM K (MG. %)	TOTAL PROTEIN (%)	BLOOD GLUCOSE (MG. %)	REDUCING SUB- STANCE†
									(MG. %)
VI	0:00	98.6	4.4	10.4	2.0	18	--	81	15
	0:55	102.0	4.0	10.1	2.4	20	--	148	29
	1:15	104.4	3.8	10.1	1.8	22	--	130	11
	2:15	104.7	3.3	10.7	3.8	19	--	138	11
	3:30	102.2	4.1	10.7	1.9	19	--	146	15
	3:45	98.0	3.6	10.5	1.8	19	--	154	11
VII	0:00	97.0	4.9	11.1	3.6	--	--	150	6
	1:10	102.0	4.0	11.0	2.3	21	--	120	14
	1:30	104.2	3.4	11.3	2.0	23	--	123	3
	3:00	104.3	2.6	11.2	1.6	22	--	166	5
	3:45	102.0	2.6	11.1	1.9	21	--	166	4
	4:00	99.6	2.8	11.1	1.7	20	--	149	4
VIII	0:00	97.6	3.9	9.9	2.6	--	--	98	23
	0:45	100.0	4.1	9.5	1.5	--	--	112	14
	1:00	102.0	3.4	9.6	2.7	--	--	112	10
	1:45	106.0	2.7	9.6	2.6	--	--	124	20
	3:45	106.0	1.8	9.8	3.6	--	--	126	14
	5:45	106.0	2.2	10.0	4.0	--	--	128	29
	6:15	104.0	2.5	10.5	5.5	--	--	138	24
	6:30	102.0	2.9	10.2	4.0	--	--	128	16
	7:00	100.0	4.7	10.1	4.0	--	--	116	10
	8:05	99.2	4.9	10.5	4.0	--	--	112	6
IX	0:00	98.2	5.0	9.0	4.2	21	6.2	120	10
	0:25	100.0	4.7	10.2	2.8	18	6.2	122	15
	0:50	102.0	3.9	10.2	1.7	17	6.1	126	8
	1:00	104.0	3.6	10.2	2.0	20	6.5	131	12
	1:20	106.0	3.1	10.3	1.9	20	6.5	131	12
	2:20	106.0	3.3	10.4	1.8	18	6.3	132	13
	3:20	106.4	3.2	10.0	2.2	--	6.5	139	8
	4:20	106.2	3.4	9.5	1.9	18	6.6	156	10
	5:20	105.3	3.6	9.3	2.5	16	6.6	153	16
	5:40	102.0	4.1	10.3	1.8	16	6.1	156	11
	6:10	100.2	4.6	10.0	2.4	17	5.8	138	14
X	0:10	98.4	2.9	10.5	2.8	33	7.2	214	30
	0:30	100.0	2.6	10.5	3.8	31	7.2	123	10
	0:40	102.0	2.2	10.3	4.1	32	7.0	144	14
	1:15	104.0	1.9	10.5	--	--	6.9	134	10
	1:40	106.0	2.2	10.6	2.3	34	6.8	198	10
	3:40	106.0	1.9	10.6	3.9	36	6.8	132	6
	5:40	106.0	1.7	10.5	4.7	33	6.9	200	10
	6:05	104.0	2.1	10.7	3.7	31	7.5	192	8
	6:12	102.0	2.3	10.7	3.0	--	7.5	198	11
	6:40	100.0	3.0	10.6	5.7	--	--	184	21
	7:10	99.6	3.8	10.7	4.4	33	7.2	173	10

\*Interval following the onset of the chill.

†Total reducing substance in the blood other than glucose; results expressed in terms of milligrams per cent of glucose.

## RESULTS

*Group A.*—In fifteen of the sixteen paroxysms studied in this group the serum phosphorus level fell below the limit of normal (3.0 mg. per cent). The average minimum level attained was 1.9 mg. per cent with a range from 1.0 to 2.9 mg. per cent. The reduction in serum phosphorus was found to be progressive and minimum levels were not reached until some time after the inception of the rigor (Table I).

*Group B.*—As shown in Tables II and III, hypophosphatemia occurred in all seventeen of these patients. The average minimum level reached was 1.6 mg. per cent with a range from 0.8 to 2.7 mg. per cent. Among fourteen cases in which phosphorus levels were analyzed very early in the chill before a significant fall had occurred, the total diminution in this mineral averaged 51 per cent of the original level (range 19 to 71 per cent).

As surmised, it was found that the development of hypophosphatemia was progressive, commencing at the time of the temperature rise with the minimum level occurring after about three or four hours. Here, however, the range was wide (two to twelve hours). If the fever and serum phosphorus levels were plotted graphically (Figs. 1A and 1B), however, it was apparent that there was an inverse relationship between the two, the latter attaining its lowest level at about the time of the fever peak. Thereafter, however, the hypophosphatemia disappeared fairly rapidly despite a continued elevation or even a secondary rise in temperature. Normal blood levels were restored before the fever had wholly subsided.

Of the other blood constituents studied only glucose exhibited a consistent relationship to the changes previously noted. Since the metabolic insult of the parasitemia preceded the inception of rigor, none of the patients could be considered to be in a basal state. It was not possible, therefore, to establish a basal take-off point in this group. Nevertheless, all of the patients exhibited an elevation of blood glucose as the febrile episode proceeded. There was an obvious tendency for the glucose level to rise parallel with the fever, but this rise continued for a number of hours after the pyrexia had arrived at its peak and had begun to subside (Figs. 1A and 1B). The maximum blood sugar levels attained averaged 184 mg. per cent with a range of 138 to 250 mg. per cent. Seven cases showed values exceeding the average. From twelve of these patients urine samples were procured during and after the paroxysm, and despite the fact that in seven of them the usual renal threshold level of 180 mg. per cent was exceeded no glycosuria was detected.

In ten of the cases fractionation of blood-reducing substance was carried out. Some variation of the nonglucose fraction occurred but was inadequate in amount to account for the rise in total reducing substance. It was apparent, therefore, that the blood sugar rise observed was almost wholly the result of increase in glucose.

Despite the very significant variation in the concentration of serum phosphorus, the calcium levels remained relatively unchanged throughout the paroxysm. At times mild hypocalcemia was noted in the earlier cases sub-

jected to preliminary studies, but in none of the rigidly selected individuals was this noted. The variation in values from the inception to the termination of the episodes was a fraction of a milligram.

Serum potassium evaluations were carried out in eleven instances. Although variation did appear the values generally remained within the limits of normal (18 to 22 mg. per cent). In two cases a potassium rise to 30 and 32 mg. per cent, respectively, occurred during the period of temperature fall, but this phenomenon was not observed in the remaining nine patients.

The value of serum magnesium was determined serially in eleven patients. As with the serum potassium, such variations as were observed were usually confined within the limits of normal (1 to 3 mg. per cent). In some instances normal values were exceeded but there was no consistent relationship to the fever or other metabolites.

In ten cases total protein estimations were made. In two of these patients the protein remained unchanged. In the remaining eight an average fall of 0.8 Gm. occurred during the paroxysm (0.4 to 1.5 Gm.). In most instances a rising trend or a return to pre-existing values had appeared at the termination of the study.

In two cases blood creatine and creatinine determinations were performed. With the latter the greatest change was 0.3 mg. per cent, while with the former there was a reduction of 1 mg. at some time during the temperature rise. This alteration of about 17 per cent was transitory and values of blood creatine remained within the limits of normal (Table III).

*Group C.*—The chemical studies carried out in this group were identical with those in Group B. The results, likewise, were somewhat similar. Among these patients the serum phosphorus levels exhibited a progressive fall as the fever curve rose (Figs. 2A and 2B), although the degree of change was not quite as great. The minimum phosphorus levels ranged from 0.7 to 3.3 mg. per cent, the average being 2.3 mg. per cent. The average fall from the pretreatment level was 41 per cent and the range from 9 to 67 per cent. It appeared that the minimal level was reached somewhat more rapidly than in those cases with malarial pyrexia (Figs. 2A and 2B), the average time being two or three hours after the induction of fever. However, the return to within normal phosphorus limits was more rapid than in the patients with malarial fever. Despite the greater rapidity of phosphorus fall in this group, the rate of temperature rise was greater so that minimum phosphorus levels occurred with a lag of one or two hours after the temperature had reached its peak.

Although these patients were all in a fasting state at the onset of the fever therapy, many showed an elevated blood sugar in the initial pretreatment sample. This may have been on a psychomotor basis as many of the patients manifested apprehension on entering the fever cabinet. One patient with a fasting blood sugar level of 175 mg. per cent showed a subsequent rise to 294 mg. per cent when the body temperature was elevated. Later investigation revealed that this patient suffered from a previously unsuspected diabetes mellitus. Of the remaining nine patients all but one showed a hyperglycemia similar to that observed with malarial fever, the rise in blood sugar roughly approximating

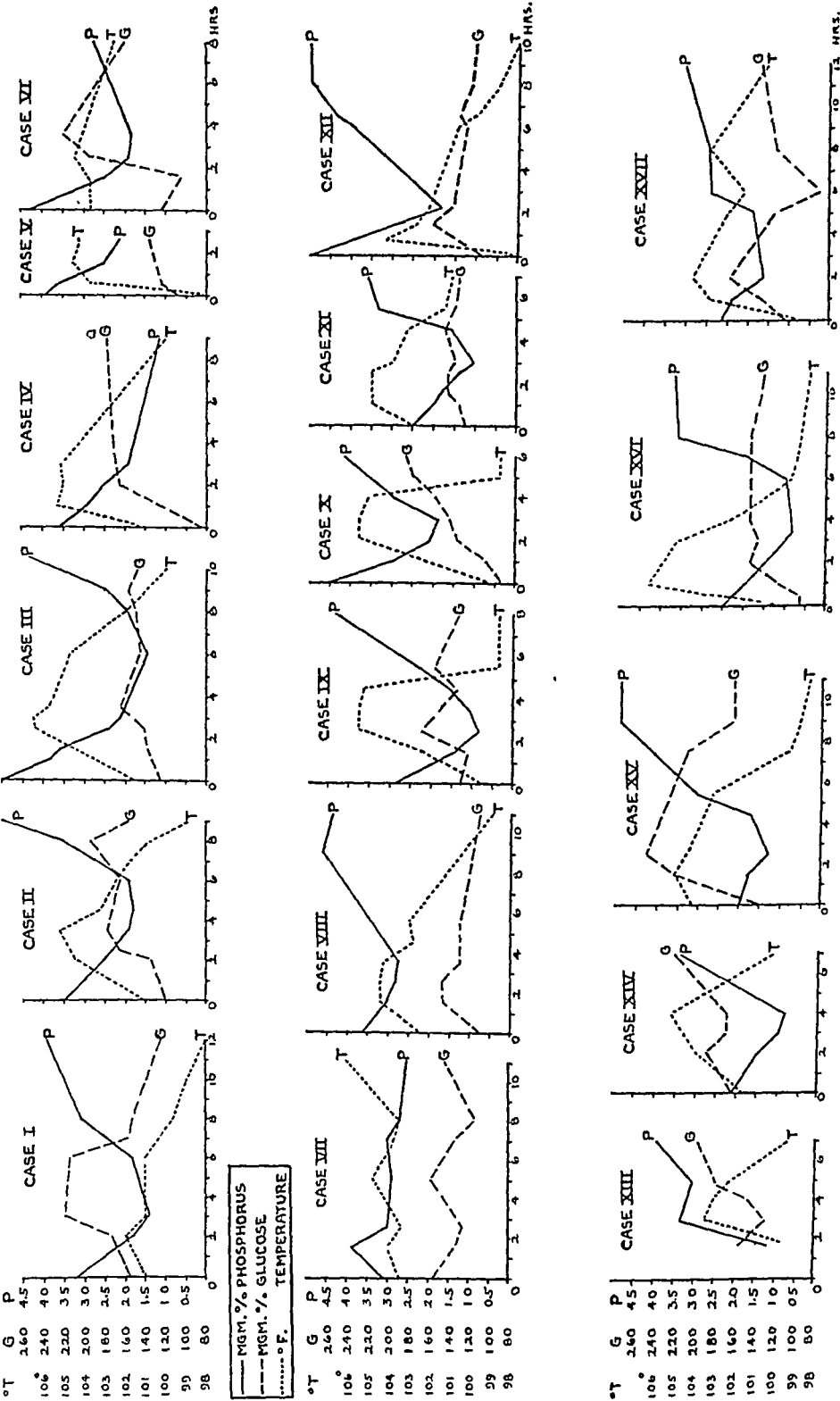


Fig. 14.—Glucose and phosphorus curves in munitin.

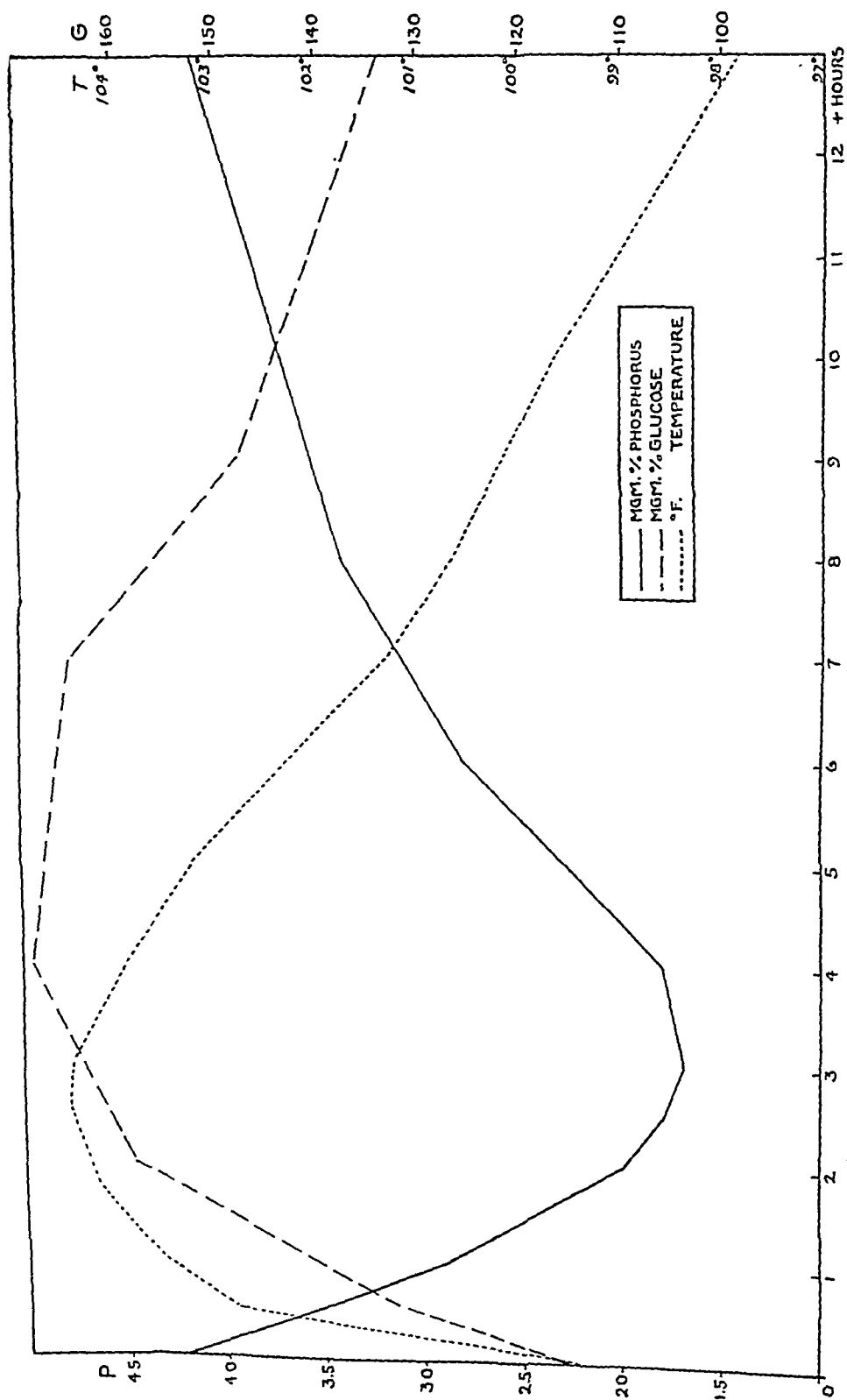


Fig. 1B.—Mean temperature and sugar and phosphorus curves during malarial paroxysm (ten cases).

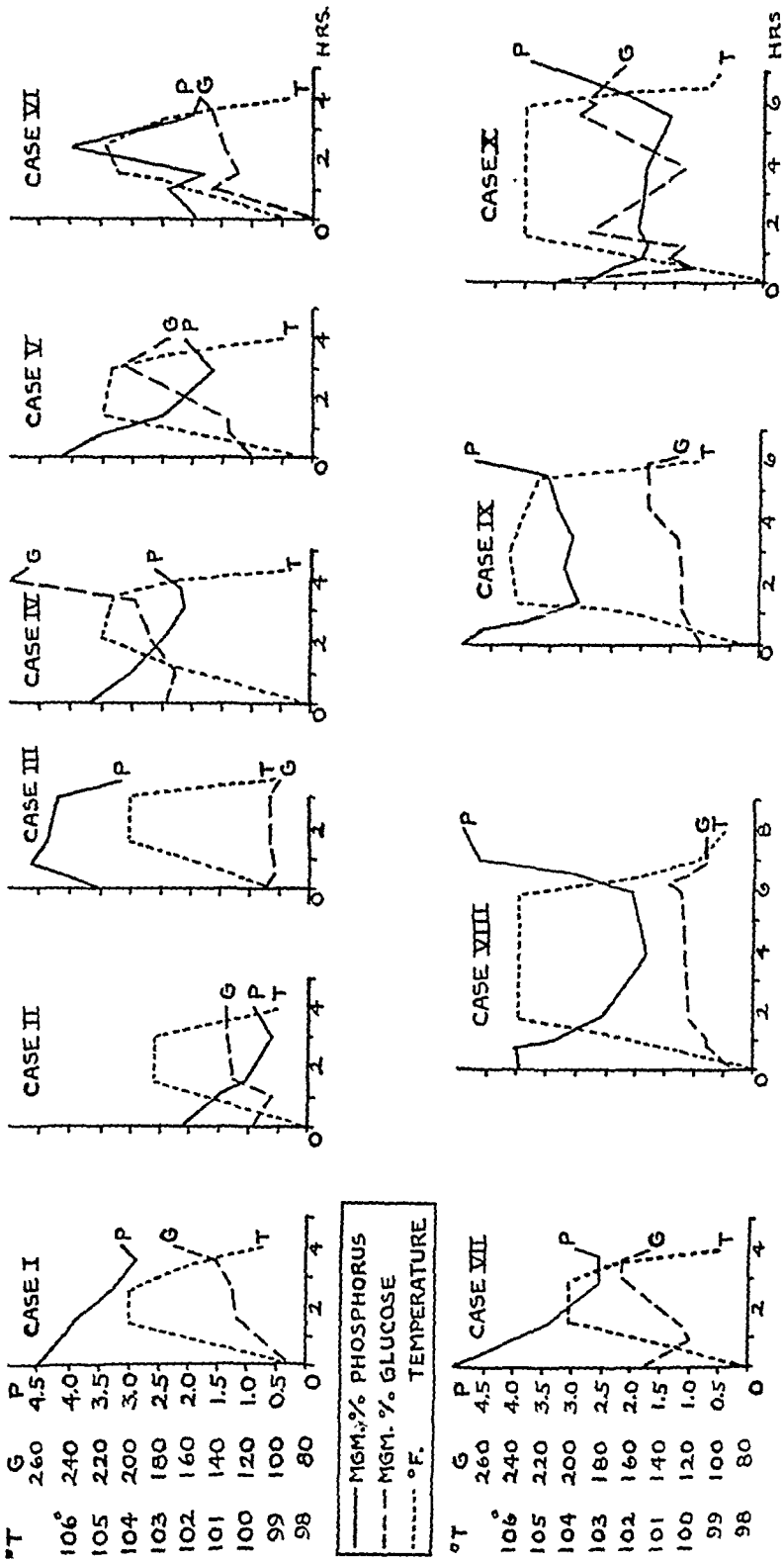
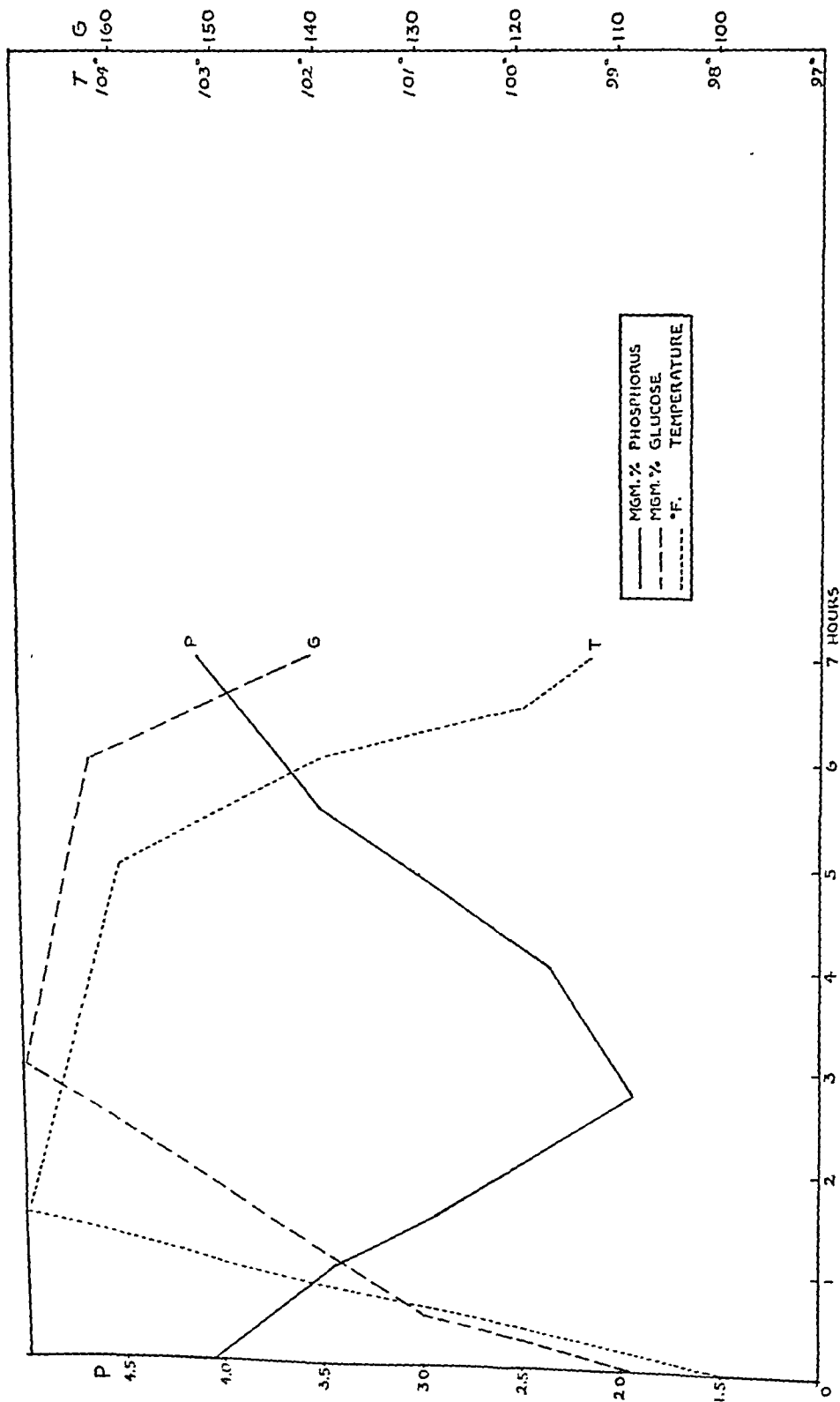


Fig. 24.—Glucose and phosphorus curves in artificially induced fever.



Pls. 2B.—Mean temperature and sugar and phosphorus curves during artificially induced fever (ten cases).



the slope of the fever curve. As in the malarial group, the rise continued and the elevation persisted for a considerable period after the temperature had returned to normal.

Exclusive of the patient with diabetes (Case IV) and the patient showing no change in blood sugar (Case III), the range of maximum levels attained was 134 to 205 mg. per cent and the average was 165 mg. per cent. Four individuals from whom urine samples were obtained exhibited no glycosuria. In two of these the blood sugar level had exceeded 180 mg. per cent.

The blood from five of these patients was analyzed for nonglucose-reducing substance and, as noted previously, such variation as occurred was inadequate to account for the rise in total reducing substance. The latter therefore consisted in the main of glucose.

Alterations in serum calcium and serum magnesium were of the order previously noted in the malarial studies. The amount and kind of variation was such as to preclude importance in the metabolic processes involved. Although one patient with paresis (Case X) showed a persistent elevation of serum potassium throughout the entire study, the fluctuation in the amount of this element in the other cases was considered insignificant. The total serum protein estimations, likewise, were of little moment. In one case there was no change, in four cases an increase of 0.4 to 0.6 Gm. per cent was noted, and in two cases there was a fall of 0.4 to 0.6 Gm. per cent.

#### DISCUSSION

A reduction in serum inorganic phosphorus was observed initially in a group of patients with malaria. The phenomenon subsequently was shown to begin toward the end of a chill when the rise in temperature was initiated and to decline to the minimum point when the fever had attained its peak. Thereafter, the phosphorus values returned rapidly to normal limits often without the reciprocal relationship to the temperature curve originally shown. The occurrence of hypophosphatemia in association with the malarial paroxysm had been noted earlier by Payne.<sup>9</sup> A similar reduction in phosphorus observed by us in a series of patients receiving artificial fever therapy paralleled those recorded previously by other investigators.<sup>4, 5, 10, 24, 50</sup>

In seeking an explanation for the hypophosphatemia, blood glucose determinations were made at comparable periods. From these studies it became apparent that the fall in serum phosphorus was attended by a rise in glucose in a manner which suggested an inverse relationship between the two. It appeared on the basis of chronological observations that the blood sugar rise occurred fairly promptly in the episode, often manifested in the initial samples procured. Return to normal values was prolonged as compared to the serum phosphorus. In many instances initial sugar values had not been re-established when the temperature reached normal. Similar observations were made during the induced febrile state. In many instances a secondary rise in temperature was attended by a further elevation in the blood sugar. However, there was no concomitant secondary reduction in serum phosphorus, the latter continuing

to approach the original normal levels without deviation. We have no explanation to offer for this paradox.

It was thought that the hyperglycemia thus produced resulted from an acceleration of metabolism<sup>8, 25, 26</sup> and the simultaneously increased muscular tonus.<sup>9</sup> It was suspected that the hypophosphatemia was linked with increased glucose utilization, a possibility further sustained by the reduction of serum phosphorus observed following the ingestion of glucose and/or the administration of insulin.<sup>27-31</sup>

It was postulated that the period characterized by the hypophosphatemia was associated with the process of phosphorylation. Glycogenolysis and glycogenesis occurring in the course of the febrile paroxysm probably were accompanied by the formation of hexosephosphate and the deposition of the latter in the blood and tissues.<sup>32</sup> The transient linkage of phosphorus with the carbohydrate component presumably resulted in hypophosphatemia. At the conclusion of the episode, the dissociation of hexosephosphate permitted the mobilization of phosphorus, the replenishing of inorganic phosphorus, and the concomitant rise of serum phosphorus to its prefebrile level. The hexose, it would seem, was reconverted thereafter to glycogen and deposited in the tissues or was decomposed further and excreted.

Some substantiation of this thesis is found in the report of Cori and Cori<sup>33</sup> who noted an increased amount of hexosephosphate in muscle during muscular activity and a disappearance of this compound when activity ceased. Of parallel interest was the recorded fall of blood sugar in diabetics following the parenteral administration of sodium phosphate.<sup>34</sup>

On the other hand, Payne<sup>9</sup> attributed the fall in serum phosphorus during induced malarial episodes primarily to the formation of phosphocreatine. This report was encountered after much of the present study was completed, but we were able to carry out similar estimations of creatine and creatinine values in two cases during spontaneous malarial chills (Table III). Blood samples were collected in these cases at frequent intervals after the onset of the chills, throughout the febrile course, and until the patients had become afebrile. We were unable to confirm Payne's observations of significant elevations in blood creatine at the time of the temperature rise. It is interesting that he had been unable to demonstrate any hyperglycemia during or following the paroxysm.

Although such urine phosphorus examinations as were carried out in our studies were crude, there is ample evidence in the literature attesting to the fact that loss of phosphorus does not occur through this medium during the febrile paroxysm.<sup>4, 5, 9</sup> Bischoff and associates<sup>4, 5</sup> attributed the hypophosphatemia and concomitant hyperglycemia encountered in experimental hyperthermia to the conversion of inorganic phosphorus to the organic state. Daly and Knudson,<sup>24</sup> however, were disinclined to accept this explanation since they noted diminution of both organic and inorganic phosphorus.

The hyperglycemia in artificial fever was considered by Hench and Slocumb<sup>25</sup> to be due to hemoconcentration. This thesis obviously is not correct in view of the concomitant hypophosphatemia and hyperglycemia and the evi-

dence of mild hemodilution shown by the diminished total protein values (Tables II to IV). Petersen<sup>11</sup> observed a diminution of blood sugar during the period of temperature rise in malaria with an elevation after the temperature had fallen. This discrepancy remains unexplained since it occurred in not a single one of the cases we observed. Kirstein and Bromberg<sup>10</sup> collected blood samples before induction and after subsidence of artificial fever. They studied no samples during the febrile period but recorded both increased glucose and diminished phosphorus after the hyperthermia. These authors considered the elevated sugar as evidence of increased glucose utilization by the tissues and also as basis for the serum phosphorus depletion. It seems reasonable to assume that results of a higher order of magnitude would have been obtained by Kirstein and Bromberg had they procured samples during the period of actual pyrexia.

Fenn,<sup>36</sup> reviewing the literature on potassium metabolism, remarked on the diminution of vascular tonus concomitant with a progressive loss of potassium and a rise in sodium during muscular activity. In our own studies of the serum potassium it did not appear that the level of this substance reflected the increased muscular activity during the rigor. For the most part the serum potassium varied only within normal limits. A few of the patients with malaria (Table II), as previously noted, showed an abrupt rise during the subsidence of the fever. One patient with paresis exhibited an inordinately high potassium level before fever was induced; this was not modified by the hyperpyrexia. None of these observations lend themselves to satisfactory explanation. A gradual elevation of serum potassium in malaria was reported by Zwemer and co-workers<sup>37</sup> and Stevenson,<sup>38</sup> the peak of rise being attained at the onset of the chill with a subsequent return to normal thereafter. Honaga<sup>13</sup> observed an increase of potassium in the blood at the height of the fever in several pathologic states with a return to normal during convalescence. On the basis of our own observations it did not appear that conclusions regarding the role of this electrolyte in fever were warranted.

The inverse relationship of serum magnesium to calcium and phosphorus and its action on the neuromuscular system<sup>39-41</sup> led to investigation of its variations in the febrile state. In both the spontaneous and induced fevers such variations as appeared, exhibited no consistent pattern. Peters and Van Slyke<sup>42</sup> have recognized this variability but were unable to offer an explanation for it. It is possible, as they suggested, that the methods for analysis used were sufficiently crude to permit such inconsistency. The magnesium values as we have observed them appear unrelated to the changes noted in other metabolites. For the most part their ranges were within normal limits.

Utilizing the amount of the total protein as an index of hydremia, it appeared that there was indeed some hemodilution of minor degree during the malarial paroxysm since the values of this substance fell rather uniformly at the febrile fastigium and rose again when the fever had subsided. Similar changes, however, were not noted in the patients with artificial fever. Certainly, there was no indication that the hyperglycemia noted was attributable to hemoconcentration as suggested by Simpson<sup>43</sup> and Hench and Slocumb.<sup>35</sup>

Studies upon other constituents of the blood revealed only incidental information. Determinations of the nonglucose-reducing substances concomitantly with the total blood-reducing substances showed that the rise in the latter was indeed the result of glucose per se and not one of the other associated compounds (ascorbic acid, cysteine, glutathione, uric acid). There was a remarkable constancy of serum calcium values which remained almost wholly unchanged throughout the febrile episode.

Studies with the cephalin-cholesterol flocculation<sup>44</sup> and thymol turbidity<sup>45</sup> tests were carried out simultaneously with those previously recorded. As had been noted previously,<sup>46-48</sup> the former were often positive in malaria. We obtained negative results in the artificial fever group. It is of interest to record that many of the patients with malaria showed positive flocculation and turbidity tests before and during the paroxysm. Others, however, showed a positive cephalin flocculation during the febrile period, the tests becoming negative when the temperature became normal. In these cases the thymol turbidity tests were negative throughout. The results of this phase of the study will be reported in detail subsequently in conjunction with a larger series of cases of miscellaneous nature.<sup>49</sup>

#### SUMMARY

1. Preliminary studies on patients with malarial fever (eighteen cases) revealed the development of transient hypophosphatemia during the febrile paroxysm.

2. Serum inorganic phosphorus, calcium, magnesium, potassium, serum protein, blood glucose, and nonglucose-reducing substance were determined at various intervals during the febrile episodes in patients with benign tertian malaria (seventeen cases).

3. Similar studies were carried out on patients with artificial fever therapy (ten cases).

4. There was a marked fall in serum phosphorus attended by a significant rise of the blood glucose in all patients with malaria and in the majority of fever therapy cases so studied.

5. It was believed that the formation and deposition of hexosephosphate in the tissues was the basis for the hypophosphatemia.

6. Hyperglycemia appeared early in fever and was subsequently followed by diminution in inorganic serum phosphorus.

7. No significant alterations were observed in the serum calcium, potassium, magnesium, creatine, or creatinine.

8. The serum protein showed a slight fall during the malarial fever but remained unchanged in artificial fever.

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## REFERENCES

1. Beck, O.: Untersuchungen zum Fieberstoffwechsel, *Klin. Wehnschr.* 4: 2276, 1925.
2. Cohn, H.: Experimentelle Hyperglykämie im Fieber, *Ztschr. f. d. ges. exper. Med.* 53: 878, 1927.
3. Cohn, T., and Houget, J.: Le métabolisme des glucides, lipides, et protides au cours d'hyperthermies, *Compt. rend. Soc. de biol.* 104: 852, 1930.
4. Bischoff, F., Ullmann, H. J., Hill, E., and Long, M. L.: Studies in Hyperthermia Induced by High Frequency Electric Current, *J. Biol. Chem.* 85: 675, 1930.
5. Bischoff, F., Maxwell, L. C., and Hill, E.: The Acid-Base Equilibrium in Hyperthermia Induced by Short Radio Waves, *J. Biol. Chem.* 90: 331, 1931.
6. Adachi, K.: Experimentelle Untersuchungen über den intermediären Stoffwechsel im Muskel bei Fieber; Phosphorsäure- und Harnsäurestoffwechsel im Muskel bei experimentellem Fieber, *Tohoku J. Exper. Med.* 20: 107, 1932.
7. Grant, W. H., and Medes, G.: Creatinine Clearance During the Hyperthermia of Diathermy and Fevers, *J. LAB. & CLIN. MED.* 20: 345, 1934.
8. Kopp, I.: Metabolic Rates in Therapeutic Fevers, *Am. J. M. Sc.* 190: 491, 1935.
9. Payne, W. W.: Phosphate and Creatine Metabolism in Fever, *Biochem. J.* 29: 1310, 1935.
10. Kirsten, M. B., and Bromberg, L.: Effect of Fever Therapy on Carbohydrate Metabolism, *J. LAB. & CLIN. MED.* 25: 7, 1939.
11. Petersen, W. F.: Blood Sugar During Crisis of Malaria Fever, *Proc. Soc. Exper. Biol. & Med.* 23: 753, 1926.
12. Emerson, K., Jr., and Dole, V. P.: Electrolyte, Water, and Nitrogen Metabolism in Acute *P. vivax* Malaria, *Malaria Report No. 247*, Sept. 22, 1944. (Board for the Coordination of Malarial Studies—*Restricted*.)
13. Honaga, S.: Clinical and Experimental Studies on the Metabolism of Calcium, Potassium, and Phosphorus During Fever, *Jap. J. Exper. Med.* 18: 255, 1940.
14. Kuttner, T. T., and Cohen, H. R.: Micro Colorimetric Studies. I. A Molybdic Acid, Stannous Chloride Reagent; the Micro Estimation of Phosphate and Calcium in Pus, Plasma, and Spinal Fluid, *J. Biol. Chem.* 75: 517, 1927.
15. Kuttner, T. T., and Lichtenstein, L.: Estimation of Phosphorus: Molybdic Acid—Stannous Chloride Reagent, *J. Biol. Chem.* 86: 671, 1930.
16. Roe, J. H., and Kahn, B. S.: The Colorimetric Determination of Blood Calcium, *J. Biol. Chem.* 81: 1, 1929.
17. Denis, W.: The Determination of Magnesium in Blood, Plasma, and Serum, *J. Biol. Chem.* 52: 411, 1922.
18. Breh, F., and Gaebler, P. H.: The Determination of Potassium in Blood Serum, *J. Biol. Chem.* 87: 81, 1930.
19. Peters, J. P., and Van Slyke, D. D.: *Quantitative Clinical Chemistry*, Vol. 1, Baltimore, 1932, Williams & Wilkins Company, p. 753.
20. Folin, O., and Wu, H.: A Simplified and Improved Method for the Determination of Sugar, *J. Biol. Chem.* 41: 367, 1920.
21. Folin, O.: A New Blood Sugar Method, *J. Biol. Chem.* 67: 357, 1926.
22. Somogyi, M.: Reducing Non-Sugars and True Sugar in Human Blood, *J. Biol. Chem.* 75: 33, 1927.
23. Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Jr., Hamilton, P. B., and Archibald, R. M.: The Copper Sulfate Method for Measuring Specific Gravities of Whole Blood and Plasma, *Bumed News Letter of U. S. Navy*, June, 1943.
24. Daly, C. A., and Knudson, A.: Acid-Base Equilibrium and Phosphorus Metabolism in Hyperthermia, *J. Biol. Chem.* 97: 57, 1932.
25. Perera, G. A.: Clinical and Physiologic Characteristics of Chill, *Arch. Int. Med.* 68: 241, 1941.
26. Baer, D. P., Cecil, R. L., and DuBois, E. F.: Temperature Regulation After Intravenous Injection of Proteose and Typhoid Vaccine, *Arch. Int. Med.* 29: 608, 1922.
27. Fiske, C. H.: The Role of Phosphoric Acid in Carbohydrate Metabolism, *Proc. Soc. Biol. Chem.*—*J. Biol. Chem.* 41: 59, 1920.
28. Perlzweig, W. A., Latham, E., and Keefer, C. S.: The Behavior of Inorganic Phosphorus in the Blood and Urine of Normal and Diabetic Subjects During Carbohydrate Metabolism, *Proc. Soc. Exper. Biol. & Med.* 21: 33, 1923.
29. Pollack, H., Millet, R. F., Essex, H. E., Mann, F. C., and Ballman, J. L.: Serum Phosphate Changes Induced by Injection of Glucose Into Dogs Under Various Conditions, *Am. J. Physiol.* 110: 117, 1934.
30. Free, A. H., and Leonards, J. R.: Serum Phosphorus Changes During the Absorption and Metabolism of Glucose, Galactose, and Xylose, *J. Biol. Chem.* 149: 203, 1943.
31. Bollinger, A., and Hartman, F. W.: Observations on Blood Phosphates as Related to Carbohydrate Metabolism, *J. Biol. Chem.* 64: 91, 1925.

32. Kerr, S. E., and Blish, M. E.: The Effect of Insulin on Phosphorus Compounds of Muscle, *J. Biol. Chem.* 97: 11, 1932.
33. Cori, G. T., and Cori, C. F.: Changes in Hexosephosphate, Glycogen and Lactic Acid During Contraction and Recovery of Mammalian Muscle, *J. Biol. Chem.* 99: 493, 1933.
34. Friedlander, K., and Rosenthal, W. G.: Ueber den Einfluss des Phosphorsäureions auf den Blut- und Harnzucker des normalen und des diabetischen Organismus, *Arch. f. exper. Path. u. Pharmacol.* 112: 65, 1926.
35. Hench, P. S., and Slocumb, C. H.: Results of Fever Therapy for Gonorrheal Arthritis, Chronic Infectious (Atrophic) Arthritis, and Other Forms of "Rheumatism," *Proc. Staff Meet., Mayo Clin.* 10: 202, 1935.
36. Fenn, W. O.: The Role of Potassium in Physiological Processes, *Physiol. Rev.* 20: 377, 1940.
37. Zwemer, R. L., Sims, E. A. H., and Coggeshall, L. T.: The Plasma Potassium Level During Malarial Infection in Monkeys and Man, *Am. J. Trop. Med.* 26: 687, 1940.
38. Stevenson, Dwight S.: The Use of Calcium Gluconate in the Treatment of Malarial Chills, *Puerto Rico J. Pub. Health & Trop. Med.* 19: 602, 1944.
39. Schmidt, C. L. A., and Greenberg, D. M.: Occurrence, Transport, and Regulation of Calcium, Magnesium, and Phosphorus in Animal Organism, *Physiol. Rev.* 15: 297, 1935.
40. Shohl, A. T.: Mineral Metabolism—Calcium and Magnesium, *Ann. Rev. Biochem.* 2: 207, 1933.
41. Sjollem, B.: Nutritional and Metabolic Disorders in Cattle, *Nutrition Abstr. & Rev.* 1: 621, 1932.
42. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry, Vol. 2, Baltimore, 1932, Williams & Wilkins Company, p. 863.
43. Simpson, W. H.: Influence of Artificial Fever on Chloride Metabolism, *J. A. M. A.* 100: 67, 1933.
44. Hanger, F. M.: Serological Differentiation of Obstructive From Hepatogenous Jaundice by Flocculation of Cephalin-Cholesterol Emulsions, *J. Clin. Investigation* 18: 261, 1939.
45. MacLagan, N. F.: Thymol Turbidity Test; a New Indicator of Liver Dysfunction, *Brit. J. Exper. Path.* 25: 234, 1944.
46. Mirsky, I. A., von Brecht, R., and Williams, L. D.: Hepatic Dysfunction in Malaria, *Science* 99: 20, 1944.
47. Sabin, A. B., Philip, C. B., and Paul, J. R.: Phlebotomus (Sandfly) Fever, *J. A. M. A.* 125: 693, 1944.
48. Bronstein, L. H., and Reid, R. D.: The Cephalin-Cholesterol Flocculation Test in Malaria, *Proc. Soc. Exper. Biol. & Med.* 60: 140, 1945.
49. Steinberg, A.: Desoxycholic Acid—Cholesterol Flocculation Test and Its Comparison With Other Tests of Liver Dysfunction. In preparation.
50. Danielson, W. H., Stecher, R. M., Muntwyler, E., and Myers, V. C.: The Acid-Base Balance of the Blood Serum in Hyperthermia, *Am. J. Physiol.* 123: 550, 1938.

# NORMAL SEDIMENTATION RATES IN ACTIVE PULMONARY TUBERCULOSIS

A STUDY OF 1,066 CASES IN A NAVAL TUBERCULOSIS CENTER

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**E**RYTHROCYTE sedimentation tests long have been of value in determining the activity of tuberculous lesions. Like many diagnostic aids, however, the test has been abused and misinterpretations occasionally have caused loss of faith<sup>1</sup> in a worth-while procedure. Recent experience in a large naval tuberculosis center (Sampson Naval Hospital) has provided opportunity to observe the use and abuse of sedimentation rates for the diagnosis and evaluation of pulmonary tuberculosis.

Patients admitted to this center offered an unusually good clinical history of the infection. Each man in the Navy carries with him a so-called health record in which is entered full reports of any medical attention tendered him while in service. Thus, each patient brought with him a fairly complete medical history containing reports of previous medical studies, x-rays, and laboratory procedures.

In a rather alarming number it was seen that men with active lesions had been under observation at a recent date because of their pulmonary infiltration. Studies during the previous admissions resulted in the diagnosis of inactive disease with consequent "back to duty" discharge. The men often returned later, however, with more advanced lesions. A study of such records indicates the continuation of a fairly widespread belief that active tuberculosis always will be reflected by an increased sedimentation rate.<sup>2-5</sup>

On the basis of the foregoing considerations, a study was made of 1,066 patients with active pulmonary disease admitted to the Sampson Naval Hospital between January, 1945, and March, 1946. They were all essentially new patients never having been treated previously for tuberculosis except at the activity which forwarded them to the tuberculosis center. In some instances bed rest of varying degrees and duration had preceded their admission, but this did not prevent their inclusion in the study if other findings indicated currently active pulmonary tuberculosis.

The sedimentation rate of each patient was determined within the first few days after admission by the Cutler procedure.<sup>6</sup> Normal limits of sedimentation in one hour by this method are 2 to 8 mm. for men and 2 to 10 mm. for women. No correction is necessary for hematocrit values.<sup>3, 7, 8</sup> The Cutler method has been found to compare favorably with other sedimentation procedures.<sup>9, 10</sup>

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From the group of 1,066 cases, patients were selected in whom the diagnosis of active pulmonary tuberculosis was relatively certain. The criteria for this diagnosis were as follows: (1) an active pulmonary lesion by x-ray examination; (2) the presence of tubercle bacilli on three separate occasions in the sputum and/or gastric washings by smear and/or culture; and (3) no evidence of pleural fluid, pneumothorax, thoracoplasty, and no tuberculosis or other disease elsewhere in the body.

There were 300 cases meeting these qualifications. Of these, 61 (or 20.3 per cent) had sedimentation rates within the normal range (Table I).

TABLE I

GROUPS	NUMBER OF CASES	NUMBER OF CASES WITH NORMAL SEDIMENTATION RATE	PER CENT OF CASES WITH NORMAL SEDIMENTATION RATE
I	300	61	20.3
(a) and (b)			
II	240	126	52.5
(a) and (c)			
I and II	540	187	34.6
III	80	42	52.5
(a) and (d)			
I, II, and III	620	229	36.9

(a) Active pulmonary lesion by x-ray examination with no evidence of tuberculosis or other disease elsewhere in the body.

(b) Tubercle bacilli in the sputum and/or gastric washings by smear and/or culture on three occasions.

(c) Tubercle bacilli in the sputum and/or gastric washings by smear and/or culture on one or two occasions.

(d) No positive bacteriologic findings but definite cavitation by x-ray examination.

A second group was then selected in which all the previously mentioned criteria were fulfilled, except that a positive smear and/or culture of sputum and/or gastric washings was found on only one or two occasions. There were 240 cases meeting these qualifications, making a total, with the first group, of 540 cases. Of these, 187 (or 34.6 per cent) had sedimentation rates within the normal range.

Finally, a group of cases was added in which all these criteria were fulfilled, except that no positive bacteriologic findings were recorded. Instead, these patients had definite cavitation as determined by the roentgenologist, mostly on stereoscopic examinations. Cases in which cavitation was only suspected were not included.

The final group of 80, when added to the first two groups, made a total of 620 cases. Of all these, 229 (or 36.9 per cent) had sedimentation rates within the normal range.

The remaining 446 cases were complicated by pleural effusion, pneumothorax, questionable x-ray shadows, or lack of positive bacteriology. No unchallenged conclusions could be drawn from sedimentation rates in this group.

#### DISCUSSION

The incidence of normal sedimentation rates in active pulmonary tuberculosis in this series is strikingly large and might be difficult to accept if other examiners had not reported comparable results.



Thiele<sup>11</sup> has found that 25 per cent of open tuberculosis, 85 per cent of the cirrhotic type, 68.3 per cent of the cirrhotic-productive type, 42.3 per cent of the productive type, and 47 per cent of the cavernous type of tuberculosis have normal sedimentation rates. Leitner<sup>12</sup> reported normal values in 32.1 per cent, Pinner<sup>13</sup> in 40 per cent, and Bobrowitz<sup>14</sup> 45 per cent normal sedimentation rates in minimal active cases. Overholt and Wilson<sup>15</sup> state categorically that "the vast majority of early lesions reflect no change in sedimentation rates."

Berg<sup>16</sup> has reported eighteen cases, Banyai and Caldwell<sup>17</sup> one hundred and eleven, Tillisch<sup>18</sup> nineteen, and Breuhaus<sup>19</sup> seven instances of normal sedimentation rates with active disease, the last in the face of advancing cavitation. Banyai and Anderson<sup>20</sup> stated that 7.35 per cent of cases with active tuberculosis had normal sedimentation rates, Sopp<sup>21</sup> that 6.6 per cent of dispensary patients, and Rest<sup>22</sup> that nearly one-quarter of demonstrable cases gave similar readings over a long period of time.

Pessar and Hurst<sup>23</sup> reported that 18 per cent of men and 12 per cent of women with active tuberculosis gave lower than expected values. De Cecio and Elwood<sup>8</sup> found that in 224 patients admitted to the center with normal sedimentation rates, 61 per cent had active disease.

Several factors might be considered to explain the relatively high proportion of normal sedimentation rates in patients at the tuberculosis center. Many of these men were found to have roentgenologic evidence of tuberculosis on routine discharge examination and therefore were hospitalized with few or no symptoms of the disease. Also, the short previous hospitalization (average estimated at seven weeks) before admission to the Sampson Naval Hospital may have allowed the secondary inflammatory reactions to subside. Some observers believe that secondary reactions are chiefly responsible for the elevation of the sedimentation rate.

It seems logical to postulate that, as tuberculosis case-finding programs are expanded and more patients are found before the onset of secondary reactions with symptoms, normal sedimentation rates may also be found more frequently.

A study of active cases at the center according to the degree of anatomic involvement (by National Tuberculosis Association standards,<sup>24</sup> is interesting. In the first group of 300 relatively certain cases of active pulmonary disease, 20.9 per cent were classified as minimal, 55 per cent moderately advanced, and 24.1 per cent far advanced. Of the cases with normal sedimentation rates in that group, 44.4 per cent were minimal, 48.2 per cent moderately advanced, and 7.4 per cent far advanced. In this last 7.4 per cent was included one patient with advancing miliary disease who had a repeatedly normal sedimentation rate.

In these three classifications the anatomic involvement was roughly paralleled by the sedimentation rates, as has been found by others including Levinson<sup>25</sup> and Wrenn.<sup>26</sup> The average values of the 300 "relatively certain" cases are plotted according to their classification in Fig. 1. It is obvious, however, from the large proportion of normal sedimentation values in all three groups, that this parallel is not of accurate diagnostic value.

The laboratory procedures in this study were done at the Sampson Naval Hospital by Navy-trained technicians. The demobilization program with its resultant personnel changes was in effect during part of this study. Thus, these results must be considered an evaluation of the sedimentation rates in tuberculosis primarily as found in this hospital.

A few of the bacteriologic studies on these cases have been included from other naval laboratories. Re-evaluation of percentages, excluding studies done elsewhere, gives no essential change in the results.

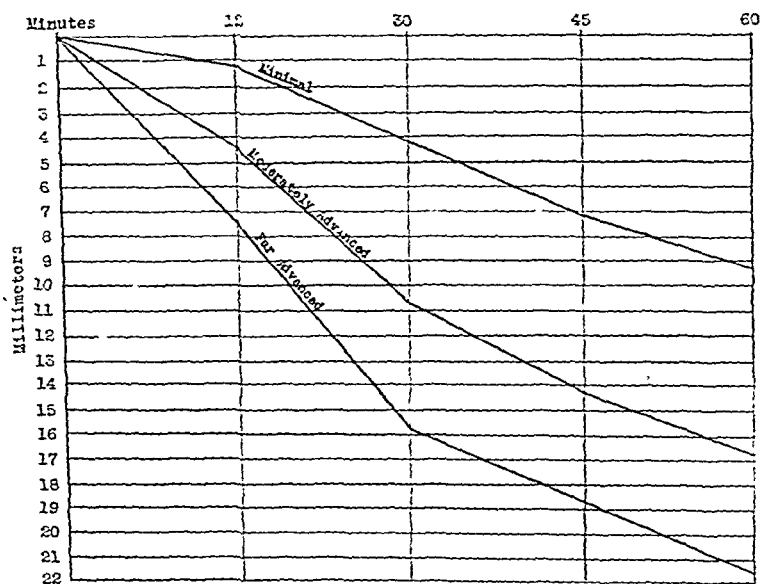


Fig. 1.—Sedimentation rates of 300 "relatively certain" cases of pulmonary tuberculosis averaged according to anatomic involvement.

### CONCLUSION

Tuberculosis per se does not always cause an elevated sedimentation rate, according to the conditions and procedure of this study. However, it cannot be denied that the test continues to be of distinct value in analyzing infectious processes, and tuberculosis is an infectious process. The sedimentation rate can continue to be extremely valuable on that basis, but on that basis only.

Except for the pathology and bacteriology of tuberculosis, our entire approach to the disease is indirect. The treatment is indirect and not specific. Diagnosis of the infection is also largely indirect. X-rays, after all, are only shadows which frequently can be misleading and often are misinterpreted and of value to a large extent from a comparative standpoint. We merely use them with these reservations as clearly in mind as possible.

The same can be said for the sedimentation test. It can, and continues to be, of help in tuberculosis until something specific, analogous to agglutination tests for other diseases, can be found. Until that time it must be used sagaciously, with all its limitations in mind.

It has been said<sup>s</sup> of the sedimentation test that it will not make a good physician from a poor one, but it will make a good one better. In tuberculosis, the physician, not the test, must make the diagnosis and determine the status of its pathology.

#### SUMMARY

1. A study of sedimentation rates in 1,066 cases of active pulmonary tuberculosis is presented.

2. Normal sedimentation rates were found in 20.8, 34.6, or 36.9 per cent of active cases, depending upon the criteria used to determine activity of the disease. This is in agreement with reports from other sources.

3. The sedimentation curve varies roughly, but not uniformly, according to the degree of anatomic involvement by the disease.

4. Tuberculosis per se does not always cause an elevated sedimentation rate. The test, however, continues to be of value in this disease if used with careful regard for its limitations.

#### REFERENCES

1. Lewis-Fanning, E., and Myers, M.: Prognostic Value of the Blood Sedimentation Rate in Pulmonary Tuberculosis, *Brit. M. J.* 2: 125, 1942.
2. Cutler, J. W., and Cohen, L.: The Blood Sedimentation Test as a Routine Procedure in the Tuberculosis Dispensary, *Am. Rev. Tuberc.* 21: 347, 1930.
3. Kaminsky, J., and Davidson, D. L.: Observations on the Red Cell Sedimentation in Pulmonary Tuberculosis, *Am. Rev. Tuberc.* 26: 282, 1932.
4. Morriss, W. N.: The Value of Erythrocyte Sedimentation Determinations in Pulmonary Tuberculosis, *Am. Rev. Tuberc.* 10: 431, 1924.
5. Westergren, A.: On the Stabilitary Reaction of Blood in Pulmonary Tuberculosis, *Brit. J. Tuberc.* 15: 72, 1921.
6. Cutler, J. W.: The Graphic Presentation of the Blood Sedimentation Test, *Am. J. M. Sc.* 171: 882, 1926.
7. Cutler, J. W., Park, F. R., and Herr, B. S.: The Influence of Anemia on Blood Sedimentation, *Am. J. M. Sc.* 195: 734, 1938.
8. De Ceccio, T., and Elwood, B. J.: Erythrocyte Sedimentation, *Am. Rev. Tuberc.* 39: 748, 1939.
9. Greisheimer, E. M., Treloar, A. E., and Ryan, M.: The Inter-relation of Cutler, Linzenmeier, and Westergren Sedimentation Tests, *Am. J. M. Sc.* 187: 213, 1934.
10. Ham, T. H., and Curtis, F. C.: Sedimentation Rates of Erythrocytes, *Medicine* 17: 447, 1938.
11. Thiele, G.: Ueber den Wert der Bestimmung der Blutkörperchensenkungsgeschwindigkeit für die Diagnosen- und Prognosenstellung bei der Lungentuberkulose, *Beitr. z. Klin. d. Tuberk.* 85: 302, 1934.
12. Leitner, J.: Die Bedeutung des weissen Blutbildes im Verhältnis zur Blutkörperchensenkungsgeschwindigkeit bei der klinischen Beurteilung einzelner Formen der Lungentuberkulose, *Beitr. z. Klin. d. Tuberk.* 86: 297, 1935.
13. Pinner, M.: *Pulmonary Tuberculosis in the Adult*, Springfield, Ill., 1945, Charles C Thomas, Publisher.
14. Bobrowitz, I. D., and Dwork, R. E.: The Early Diagnosis of Minimal Pulmonary Tuberculosis, *New England J. Med.* 234: 10, 1946.
15. Overholt, R. H., and Wilson, N. J.: Silent and Masquerading Intrathoracic Lesions, *New England J. Med.* 234: 169, 1946.
16. Berg, S.: Offene Lungentuberkulose mit niedrigem bzw. normalem Blutsenkungswert, ihr Vorkommen, ihre Prognose und Therapie, *Beitr. z. Klin. d. Tuberk.* 83: 551, 1933.
17. Banyai, A. L., and Caldwell, E.: Normal Sedimentation Rates in Open Pulmonary Tuberculosis, *Am. Rev. Tuberc.* 38: 491, 1938.
18. Tillisch, A.: Quoted in Berg, S.<sup>16</sup>
19. Breuhaus, R.: Sedimentation Test in Tuberculosis, *Med. Welt.* 9: 1867, 1935.

20. Banyai, A. L., and Anderson, S. V.: Erythrocyte Sedimentation Test in Tuberculosis, *Arch. Int. Med.* 46: 787, 1930.
21. Sopp, J. W.: Die Bedeutung der Bestimmung der Blutkörperchensenkungsgeschwindigkeit in der Tuberkulosefürsorge, *Beitr. z. Klin. d. Tuberk.* 87: 86, 1935.
22. Rest, A.: The Erythrocyte Sedimentation Rate in Pulmonary Tuberculosis, *Tubercle* 19: 307, 1938.
23. Pessar, H. T., and Hurst, A.: Erythrocyte Sedimentation, *Am. Rev. Tuberc.* 38: 495, 1938.
24. Diagnostic Standards and Classifications of Tuberculosis, *Tr. Nat. Tuberc. A.*, New York, 1940, 32 pp.
25. Levinson, S. A.: The Suspension Stability Rate of Erythrocytes in Pulmonary Tuberculosis, *Am. Rev. Tuberc.* 7: 264, 1923.
26. Wrenn, H. T.: The Blood Sedimentation Test in Pulmonary Tuberculosis, *U. S. Vet. Bur. M. Bull.* 3: 703, 1927.

# EFFECT OF 3,3' METHYLENEBIS (4 HYDROXYCOUMARIN) ON VITAMIN A DEFICIENT DUCKS

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THE experimental observations of Wakin and associates<sup>1</sup> indicate that dicumarol\* in large doses may have a toxic action on certain animals prior to the onset of hypoprothrombinemia. Several studies have shown that hypoprothrombinemia resulting from dicumarol requires a latent period of approximately twenty-four hours.<sup>2-4</sup> Some dogs given very large doses of this drug become dyspneic, have convulsions, and are comatose preceding death. No hemorrhagic manifestations occurred during life nor were hemorrhages found at autopsy in these dogs.<sup>5</sup> Dogs given intravenously 40 to 60 mg. of dicumarol per kilogram of body weight usually died within an hour without showing any changes in either the prothrombin or the coagulation times.<sup>1</sup>

Richards and Cortell<sup>6</sup> in 1942 observed that guinea pigs depleted of vitamin C succumb earlier to the effects of dicumarol. They said that "a connection between vitamin C level and sensitivity to dicumarol must be considered possible." Persons dying from infections may have a low vitamin A content in the liver.<sup>7-9</sup> Apparently the mechanism of lowered resistance to infection in vitamin A deficiency is not well understood. Susceptibility may not be directly related to the deficiency, since some individuals show a normal amount of vitamin A in the presence of disease. Bessey and Wolback<sup>10</sup> have called attention to the fact that "during a prolonged deficiency, the reserve (of vitamin A) is gradually depleted, normal cell functions are suspended, and pathologic change develops."

During recent studies on malaria in ducks it was observed that normal ducklings frequently died within three or four hours following the oral administration of large doses of dicumarol. There was observed also a variation in the time of death following the oral administration of dicumarol in ducks fed a vitamin A deficient ration when compared with birds fed a standard commercial ration. These experimental observations are reported at this time.

## METHODS AND MATERIAL

Ducks used in these studies were white Pekins obtained when 1 day of age from a commercial hatchery. They were kept in small batteries and given food and water ad lib. The standard ration was duck Startena and duck Growena. The composition of the low vitamin A ration and the standard rations are as follows:

From the Department of Pathology, University of Arkansas School of Medicine.  
Aided by a grant from the John and Mary R. Markle Foundation and Eli Lilly and Company, Indianapolis, Ind.

The dicumarol was supplied by The Wm. S. Merrell Company, Cincinnati, Ohio.  
The vitamin A deficiency ration was supplied by the Ralston Purina Mills, St. Louis, Mo., through the courtesy of Dr. Homer Patrick.

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\*Dicumarol is the collective trade-mark of the Wisconsin Alumni Research Foundation and is used for 3,3' methylenebis (4 hydroxycoumarin).

## LOW VITAMIN A DUCK RATION

Mineralized salt	0.25
Puriflavin (250)	1.00
Calcium carbonate	2.00
Soybean oil meal	18.00
Wheat germ	5.00
Fish meal (60%)	6.00
Meat scraps	4.00
Brewers' yeast	5.00
Ground wheat	19.65
White corn	29.00
Ground oats	5.00
Molasses	5.00
Dry D (2,000 D)	0.1

Vitamin mixture (milligram per 100 Gm. ration)

Thiamin	0.5
Riboflavin	0.5
Choline	200.0
Folic acid	0.5
Niacin	5.0

Ration I prepared Jan. 2, 1946; II, Jan. 28, 1946; and III, March 10, 1946.

The following data are all that are available on the two commercial rations:

## DUCK STARTENA RATION

Crude protein not less than	20.0%
Crude fat not less than	3.5%
Crude fiber not more than	6.5%
Nitrogen-free extract not less than	44.0%

Ingredients: Corn meal, ground oats, wheat bran, alfalfa leaf meal, meat scrap, fish meal, soybean oil meal, liver meal, corn gluten meal, wheat standard middlings, wheat germ, riboflavin supplement, vitamin A and D feeding oils, D activated animal sterol, 2% calcium carbonate (limestone), .25% iodized salt, .01% manganese sulfate.

## DUCK GROWENA RATION

Crude protein not less than	18.0%
Crude fat not less than	3.5%
Crude fiber not more than	6.0%
Nitrogen-free extract not less than	48.0%

Ingredients: Meat scrap, soybean oil meal, corn gluten meal, fish meal, liver meal, ground oats, corn meal, wheat bran, wheat gray middlings, wheat germ, riboflavin supplement, vitamin A feeding oil, D activated animal sterol, 2% calcium carbonate (limestone), .25% iodized salt. .01% manganese sulfate.

Vitamin A (alphalin\*) was used to supplement these rations. The contents of one capsule (25,000 units) was dropped into the mouth the younger birds and the entire capsule was given by mouth to ducks over 10 days of age. The vitamin A was given at intervals of one to three days during the time of the experiment.

Ducks were selected from the groups at different intervals and their liver was removed immediately following decapitation. The tissue was kept frozen until the vitamin A content was determined by the antimony trichloride reaction.†

The dicumarol was given by mouth. The quantity varied; in each experiment, however, a group of ducks from one hatching was used and an equal-sized dose of dicumarol was given to each bird. The amount is recorded in each experiment.

The ducks were carefully observed during the time of the experiment for the appearance of clinical manifestations. The number that died and the time of death were recorded. The average weight of the ducks used in each experiment is given in Table I.

\*Eli Lilly's preparation of vitamin A.

†These tests were made by Dr. Homer Patrick of the Ralston Purina Mills, St. Louis, Mo.

TABLE I. LETHAL EFFECT OF DICUMAROL ON VITAMIN A DEFICIENT DUCKS

EX- PERI- MENT	NUM- BER OF DUCKS IN GROUP	AGE IN DAYS	AVER- AGE WT. OF GROUP (GM.)	RATION	EXPERIMENTAL DAYS—AMOUNT OF DICUMAROL					
					0	1	2	3	4	5 6
A	5	15	360	Vitamin A deficiency Ration II*	50†	75	100	50		
					2‡	3	4	5		
	5	15	374	Standard ration	50	75	100	50		
B					0	0	0	0		
	2	17	630	Vitamin A deficiency Ration II	100	50				
					1	1	1			
C	5	17	477	Standard ration	100	50				
					0	0	0			
	12	17	469	Vitamin A deficiency Ration III	50	50	100	200	200	
D					0	0	2	5	10	10
	10	17	489	Standard ration	50	50	100	200	200	
					0	0	1	1	8	9 9
E	12	17	469	Vitamin A deficiency Ration III	100	100	150	200	200	
					5	5	5	6	11	12 12
	10	17	481	Standard ration	100	100	150	200	200	
F					1	1	1	3	7	7 7
	8	18	570	Standard ration Plus vitamin A	100	150	150	150	150	
					1	2	2	2	2	2 2
G	10	18	500	Standard ration	100	150	150	150	150	
					0	5	5	6	8	8 8
	9	18	563	Standard ration Plus vitamin A	150	150	150	150	150	
H					3	3	3	3	3	4 4
	10	18	504	Standard ration	150	150	150	150	150	
					8	8	8	8	8	8 8
I	4	28	1,065	Vitamin A deficiency Ration I	250	300				
					0	3	3			
	4	28	1,121	Vitamin A deficiency Ration I plus vitamin A	250	300				
J					0	0	0			
	12	18	496	Vitamin A deficiency Ration III	100	100	100	150	150	
					1	1	2	5	8	8 8
K	12	18	500	Vitamin A deficiency Ration III plus vita- min A	100	100	100	150	150	
					1	1	1	1	10	10 10
	12	18	584	Standard ration	100	100	100	150	150	
L					0	0	0	5	5	6 8
	3	40	1,476	Vitamin A deficiency Rations I and II	300	300				
					0	1	2			
M	3	40	1,693	Vitamin A deficiency Rations I and II plus vitamin A	300	300				
					1	1	1			
	1	40	1,685	Standard ration	300	300				
N					0	0	0			

\*Composition of different rations is given in body of text.

†Milligrams of dicumarol given each day is shown as the upper figure.

‡Total number of deaths is shown as the lower figure.

## EXPERIMENTAL EFFECT OF DICUMAROL ON DUCKS FED A VITAMIN A DEFICIENT RATION

A total of 149 ducks, as shown in Table I, were used in these experiments. In Experiment A, ten ducks were used, five of which were fed the vitamin A deficient ration II and five the standard rations. When 15 days of age these birds were given daily 50, 75, 100, and 50 mg. of dicumarol. On the third day each of the five birds fed the vitamin A deficient ration was dead; the five fed the standard rations had survived.

In Experiment B, seven ducks were used, two of which were fed the vitamin A deficient ration II and five the standard ration. When 17 days of age these ducks were given daily 100 and 50 mg. of dicumarol. On the second day one of the two ducks fed the vitamin A deficient ration was dead; the five fed the standard rations had survived.

In Experiment C, twenty-two ducks were used, twelve of which were fed the vitamin A deficient ration III and ten the standard rations. When 17 days of age these ducks were given daily 50, 50, 100, 200, and 200 mg. of dicumarol. On the third day five from the group of twelve fed the vitamin A deficient ration were dead, while only one from the group of ten fed the standard rations had died. On the sixth day ten ducks from the group of twelve fed the deficient ration were dead; nine were dead from the group of ten fed the standard rations.

In Experiment D, twenty-two ducks were used, twelve of which were fed the vitamin A deficient ration III and ten the standard rations. When seventeen days of age these birds were given daily 100, 100, 150, 200, and 200 mg. of dicumarol. Six hours following the first dose of dicumarol five ducks from the group of twelve fed the deficient ration were dead while only one had died in the group fed the standard rations. On the fourth day eleven of the twelve ducks fed the deficient ration had died, while seven were dead from the group of twelve fed the standard rations.

In these four experiments, seventeen ducks (54.8 per cent) were dead by the third day from the group of thirty-one fed the vitamin A deficient rations. Only four (13.3 per cent) were dead from the group of thirty ducks fed the standard rations for an equal period.

#### EFFECT OF DICUMAROL ON DUCKS GIVEN A HIGH VITAMIN A RATION

In this experiment, forty-five ducks were used.

In Experiment E, eight ducks were fed the standard rations supplemented with vitamin A, while ten ducks were fed only the standard rations. On the eighteenth day these birds were given daily 100, 150, 150, 150, and 150 mg. of dicumarol. Six hours following the second dose of dicumarol five of the ten ducks fed the standard rations had died, while only two from the group of eight fed the standard rations supplemented with vitamin A were dead.

In Experiment F, nineteen ducks were used, ten of which were fed the standard rations, while nine were fed the standard rations supplemented with vitamin A. On the eighteenth day of the experiment these ducks were given daily 150 mg. of dicumarol for five days. Six hours after the first dose of dicumarol was given six of the ten ducks on the standard rations had died, while only three from the group of nine on the standard rations supplemented with vitamin A were dead.

In Experiment G, eight ducks were used, four of which were fed the vitamin A deficient ration I and four the same deficient A ration supplemented with vitamin A. On the twenty-eighth day of the experiment these birds were given daily 250 and 300 mg. of dicumarol. On the second day three of the



four ducks fed the standard ration were dead; neither of the four birds fed the vitamin A deficient ration supplemented with vitamin A died.

Experiments E and F show that ducks given large amounts of vitamin A are more resistant to the acute action of dicumarol than birds of similar age fed the standard rations. In the former group seventeen ducks were used and in the latter twenty. On the third day five ducks (29.4 per cent) had died from the group fed the standard rations supplemented with vitamin A, while fourteen (70 per cent) were dead from the group fed only the standard rations.

#### EFFECT OF DICUMAROL ON DUCKS GIVEN A LARGE, MEDIUM, AND LOW QUANTITY OF VITAMIN A

In these experiments a total of forty-three ducks were used. To obtain ducks with the smallest amount of vitamin A, the vitamin A deficient rations were fed. The standard rations were fed to obtained birds with a medium quantity of vitamin A, while those with the largest quantity of vitamin A were fed the vitamin A deficient ration III supplemented with large amounts of vitamin A.

In Experiment H, thirty-six ducks were used. Three groups of twelve birds each were given one of the three rations as previously stated to obtain the three different levels of vitamin A. On the eighteenth day these ducks were given daily 100, 100, 100, 150 and 150 mg. of dicumarol. On the third day five ducks had died from the group fed the vitamin A deficient ration, five were dead from the group fed the standard rations, while only one had died from the group of twelve ducks fed the vitamin A deficient ration supplemented with vitamin A.

In Experiment I, seven ducks were used. Three of these ducks were fed the vitamin A deficient rations I and II, three were fed the vitamin A deficient rations supplemented with vitamin A, and one duck was fed the standard rations. On the fortieth day of the experiment the seven ducks were given 300 mg. of dicumarol daily for two doses. On the second day two of the three ducks fed the vitamin A deficient rations were dead; one of the three birds given the vitamin A deficient ration supplemented with vitamin A was dead; and the one bird fed the standard rations during this time survived the effects of the dicumarol.

In these two experiments fifteen ducks were fed the vitamin A deficient rations and given dicumarol; from this group seven (46.6 per cent) died. Fifteen ducks were fed the vitamin A deficient ration supplemented with vitamin A and two (13.3 per cent) died. Thirteen ducks were fed the standard rations and five (38.4 per cent) were dead by the third day.

The respiratory rate was greatly increased within two or three hours after the larger doses of dicumarol were given. This increase in the respiratory rate persisted for two or three hours during which time many of the ducks died. The ducks that survived the first large dose of dicumarol showed less severe clinical reaction on subsequent days to the same amount of dicumarol. The ducks that died appeared to be in satisfactory condition until a few minutes before death except for their rapid respiration. During the last few minutes

of life these dicumarol-treated ducks usually had one or more convulsions. Hemorrhages usually were absent in these ducks that succumb after the second and third dose of dicumarol. Some bleeding was present, however, in the tissues of the ducks that died on the fifth and sixth days of the experiment.

#### VITAMIN A CONTENT OF THE LIVER OF DUCKS

Samples from the groups of ducks were taken to determine the amount of vitamin A present in the liver. On the eighteenth day the liver from two ducks fed the vitamin A deficient ration I showed 1.5 and 14.4 U.S.P. units per gram of liver. This same deficient ration was given two other ducks and vitamin A was used to supplement it. The liver from these two ducks showed, respectively, 1,360 and 1,520 U.S.P. units per gram of liver. Two ducks were fed the vitamin A ration III and on the eighteenth day the liver from each was removed. Neither of these two birds had any vitamin A in the liver. Two ducks fed the standard rations for eighteen days had, respectively, 13.8 and 18.2 U.S.P. units of vitamin A per gram of liver tissue. The vitamin A deficient ration III was supplemented with vitamin A and fed to two ducks for eighteen days. One duck had 582 and the second 546 U.S.P. units of vitamin A in the liver.

#### WEIGHT OF VITAMIN A DEFICIENT DUCKS

The following is the average weight of groups of ducks fed the vitamin A deficient ration III, vitamin A deficient ration III supplemented with vitamin A, and the standard rations:

DUCKS	AGE IN DAYS	RATION	AVERAGE WEIGHT (GM.)
24	15	Vitamin A deficient ration	425
20	15	Standard rations	442
12	18	Vitamin A deficient ration	496
12	18	Vitamin A deficient ration plus vitamin A	500
12	18	Standard rations	584
28	20	Vitamin A deficient ration	623
20	20	Vitamin A deficient ration plus vitamin A	516

Ducks fed the vitamin A deficient rations usually were slightly lighter than those kept on the standard rations. The variation in weight of the different groups, however, was not very great.

#### DISCUSSION

The observation that ducks fed large amounts of vitamin A are relatively resistant to large doses of dicumarol is interesting since vitamin A is stored in the liver and dicumarol has been found to produce necrosis in the liver of laboratory animals.<sup>1, 6</sup> Apparently the mechanism has not been established for the development of hepatic necrosis following large doses of dicumarol. This action occurs, however, before the hypoprothrombinemia.<sup>1</sup> No specific pathologic changes have been observed in the liver of ducks given large doses of dicumarol for as long as five days. The cause of the acute death in these ducks is not known. It has been observed, however, that a 50 per cent decrease

may occur in the number of red blood cells within two to four hours following the oral administration of large doses of dicumarol. The plasma at this time does not show any hemolysis; the decrease in the hematocrit reading seems to parallel the decrease in the number of red cells. The carbon dioxide content of the plasma is approximately the same as that of normal birds. There is a decrease, however, in the carbon dioxide-combining power of the plasma of dicumarol-treated birds.<sup>12</sup> Experimental observations in our laboratory show that ducks fed the vitamin A deficient ration show no significant variations from the normal in the carbon dioxide content and the combining power of their plasma.

Since the reserve of vitamin A in the liver is low in certain hepatic diseases, it may be advisable to be cautious in the use of dicumarol in the treatment of such individuals if the experimental results as observed in this study should be duplicated in man. Organic and functional changes have been observed in the liver associated with deficiencies other than vitamin A. In chronic vitamin B complex deficiencies functional and fatty changes have been described.<sup>13, 14</sup> A decrease occurs in hepatic function when dogs are fed a black tongue diet.<sup>15</sup> The recent observations of Brunschwig and associates<sup>16, 17</sup> are interesting in that they have shown that both thioglycollic and glycollic acids afford a degree of protection against hepatic cell necrosis due to dietary deficiency and to carbon tetrachloride in the rat. "The results with thioglycollic and glycollic acids are of interest since by their use it would appear that the lipotropic factor and necrosis protecting factor are shown not to be necessarily identical."<sup>17</sup>

It is obvious that the amounts of dicumarol used in these experiments are greatly in excess of those used therapeutically in man; in preliminary experiments, however, it was ascertained that doses of this magnitude were required to obtain the results described. There may be no relation between these experimental studies and the reactions which occur in man. However, the observation that vitamin A deficient ducks are more susceptible to the lethal effect of dicumarol seems of scientific interest.

#### SUMMARY

The susceptibility of ducks to the acute lethal effect of dicumarol is influenced by the intake of vitamin A. Birds fed a ration supplemented with vitamin A are more resistant to the acute lethal effects of dicumarol than birds fed a ration low in vitamin A. The mechanism by which this effect occurs is not known.

#### REFERENCES

1. Wakin, K. G., Chen, K. K., and Gatch, W. D.: The Immediate Effects of 3,3'-Methylenebis (4 Hydroxycoumarin) on Experimental Animals, *Surg., Gynec. & Obst.* 76: 323, 1943.
2. Barker, N. W., Butt, H. R., Allen, E. V., and Bollman, J. L.: The Effect of 3,3'-Methylenebis (4 Hydroxycoumarin) on Blood Coagulation Factors, *J. A. M. A.* 118: 1003, 1942.
3. Stats, D., and Bullock, J. G. M.: Effect of a Single Dose of 3,3'-Methylenebis (4 Hydroxycoumarin) Upon Blood Coagulation in Humans, *Proc. Soc. Exper. Biol. & Med.* 50: 66, 1942.

4. Butt, H. R., Allen, E. V., and Bollman, J. L.: A Preparation From Spoiled Sweet Clover (3,3'-Methylenebis-Bis-3 (4 Hydroxycoumarin) Which Prolongs Coagulation and Prothrombin Time of Blood; Preliminary Report of Experimental and Clinical Studies, Proc. Staff Meet., Mayo Clin. 16: 388, 1941.
5. Bingham, J. B., Meyer, O. O., and Poble, F. J.: Studies on the Hemorrhagic Agent 3,3'-Methylenebis (4-Hydroxycoumarin) Am. J. M. Sc. 202: 563, 1941.
6. Richards, R. K., and Cortell, R.: Studies on the Anticoagulant 3,3'-Methylene-Bis-(4 Hydroxycoumarin) Proc. Soc. Exper. Biol. & Med. 50: 237, 1942.
7. Wolff, L. K.: On the Quantity of Vitamin A Present in the Human Liver, Lancet 223: 617, 1932.
8. Moore, T.: Vitamin A Reserve of the Human Liver in Health and Disease, Lancet 223: 669, 1932.
9. Moore, T.: Vitamin A and Carotene; the Vitamin A Reserve of the Adult Human Being in Health and Disease, J. Biol. Chem. 31: 155, 1937.
10. Bessey, O. A., and Wolback, S.: The Vitamin, American Medical Association, Chicago, 1939, p. 29.
11. Altschule, M. D.: Vitamin A Deficiency, in Spite of Adequate Diet in Congenital Atresia of Bile Ducts and Jaundice, Arch. Path. 20: 845, 1935.
12. Rigdon, R. H., and McCain, B.: Effect of 3,3'-Methylenebis (4 Hydroxycoumarin) Dicumarol on *P. lophurae* Infection in Ducks. To be published.
13. Drill, V. A., and Loomis, T. A.: Functional and Fatty Changes in Liver During Chronic Vitamin B Complex Deficiency, Proc. Soc. Exper. Biol. & Med. 61: 374, 1946.
14. Drill, V. A., Shaffer, C. B., and Leathem, J. H.: Hepatic Injury in Dogs Resulting From a Vitamin B Complex-Free Diet, Proc. Soc. Exper. Biol. & Med. 52: 328, 1943.
15. Rhoads, C. P., and Miller, D. K.: Hepatic Dysfunction in Dogs Fed Diet Causative of Black Tongue, J. Exper. Med. 67: 463, 1938.
16. Green, J., and Brunshwig, A.: Protective Action of Certain Compounds Against Dietary Hepatic Drainage in the Rat, Proc. Soc. Exper. Biol. & Med. 61: 348, 1944.
17. Brunshwig, A., Johnston, C., and Nichols, S.: Carbon Tetrachloride Injury of the Liver; the Protective Action of Certain Compounds, Proc. Soc. Exper. Biol. & Med. 60: 388, 1945.

# SOME FACTORS AFFECTING THE SUSCEPTIBILITY OF RATS TO VARIOUS BARBITURATES

## THE EFFECT OF AGE AND SEX

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THE evidence concerning the susceptibility of rats to pentobarbital sodium in regard to age and sex is controversial. Fitch and Tatum,<sup>1</sup> using a dose of 75 mg. per kilogram as the L.D.<sub>50</sub> for rats when injected intraperitoneally, concluded that there is no appreciable difference in the susceptibility of the females and males. But Moir<sup>2</sup> found that the anesthetic responses varied with sex and age as growth proceeded: at first, a greater resistance in the female was seen; later, this was followed by an equality in the response of the two sexes; finally, in the older rats, the male was consistently more resistant. Nicholas and Barron<sup>3</sup> also pointed out that adult females subjected to sodium amytal were affected more than males; in addition they observed that immature rats require a lower dose of this drug to produce anesthesia than do adult rats. In agreement with these workers, Etsten and associates<sup>4</sup> reported that newborn rats are more susceptible to pentobarbital than are adults, while Carmichael<sup>5</sup> noted that the median lethal dose of pentobarbital is less for old rats weighing 150 to 325 grams than for young animals weighing 25 to 260 grams. In the present investigation an attempt was made to re-examine this question with the hope of resolving these contradictions.

## METHODS

Three different strains of rats were employed: one from our own colony (C), another from that of the Department of Anatomy (A), and a third from the Rockland Company (R). Unless otherwise indicated in the tables in this paper, rats from our colony were used. The females were nonpregnant. All rats were kept on adequate diets, but food was withheld fifteen or sixteen hours before injecting the drugs, though water was always available.\* All barbiturate solutions were freshly prepared and were never older than three hours. The observations were made at 29° Centigrade. The sex, age, weight, strain of rats, rates of injection, and concentration of solutions are presented in the tables.

The criteria used to estimate whether the anesthesia was deep or light were the ear reflex and the posture of the animal. For the ear reflex the response of the pinna is tested with a feather cut to a fine point and inserted into the ear canal. The postural reaction is indicated by the side position, when the rat cannot assume the normal posture due to loss of the righting reflex. The

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\*The newborn rats were not fasted. Immediately before injection they were taken away from their mothers. To prevent intraperitoneal injection, a sphincter clamp was placed on the point of the needle was withdrawn. This clamp was taken off after five minutes. of the animals which died did so in ten to thirty minutes. Few deaths occurred after three hours with the doses used, and the newborn were returned to their mothers after the three-hour period.

absence of the ear reflex occurs only during deep surgical anesthesia. The interval after the ear reflex has returned, but before the righting reflex is restored, measures the period of light anesthesia.

## RESULTS AND DISCUSSION

It is shown in Table I that rats weighing 50 to 100 grams each (four to six weeks old) yield results which vary only within the error of the method in regard to the influence of sex. In the weight range of 101 to 200 grams, however, a sharp increase is observed in the duration of sleep in the females, an increase which is pronounced even at the low dose of 50 mg. per kilogram and which is still more marked with higher doses (Fig. 1). At 90 mg. per kilogram, nine of twenty-nine females died, while only

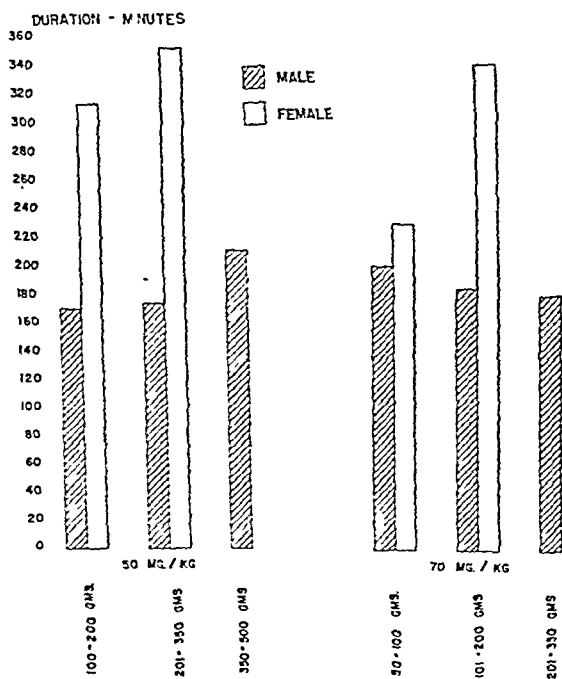


Fig. 1.—Effect of sex on duration of anesthesia. Comparative durations of side position, that is, total period of anesthesia, are presented diagrammatically. Female rats remain anesthetized for a longer period than male animals, after receiving an intraperitoneal injection of pentobarbital (1 per cent solution).

two of twenty-seven males succumbed. In the older group of rats weighing 201 to 350 grams, deaths occur even at 50 mg. per kilogram. At this dose, eleven of forty-eight males and sixteen of thirty females died. In every instance the duration of the narcosis is longer in the female than in the male. When 70 mg. per kilogram was administered to nineteen females in this weight group, all of them died (Fig. 2). Finally, although injection of the oldest males (351 to 500 grams) with 50 mg. per kilogram of pentobarbital resulted in a somewhat lower incidence of mortality (thirteen of sixty-three died) than in the group of males weighing from 201 to 350 grams, the duration of anesthesia was increased

TABLE I. EFFECT OF INTRAPERITONEAL PENTOBARBITAL ON RATS (1 PER CENT SOLUTION)

SEX	WEIGHT RANGE (GRAMS)	40 MG./KG.*			50 MG./KG.			70 MG./KG.			90 MG./KG.			120 MG./KG.		
		MOR-TALITY†	SIDE POSITION (MIN.)		MOR-TALITY	SIDE POSITION (MIN.)		MOR-TALITY	SIDE POSITION (MIN.)		MOR-TALITY	SIDE POSITION (MIN.)		MOR-TALITY	SIDE POSITION (MIN.)	
M	50 to 100															
F																
M	101 to 200															
F																
M	201 to 350	0/15	118 (81 to 133)	0/20	0/16	171 314 (161 to 202)	0/20	0/20	184 339 (124 to 266)	0/20	0/20	253 391 (178 to 388)	2/27	13/19	467 (399 to 505)	
F																
M	351 to 500															
F																

\*Dose of pentobarbital administered.

†Each fraction represents the incidence of mortality; the numerator indicates the number of animals that died and the denominator the total number of animals used.

total number of animals used.

by 22 per cent. It would have been desirable to inject more male rats in the weight range of 201 to 350 grams, but they were not plentiful in our colony. Fitch and Tatum<sup>1</sup> give 75 mg. per kilogram as the L.D.<sub>50</sub> for pentobarbital. Because the age and sex of these rats were not stated, we may point to Table I to show that this figure may be derived by employing both male and female rats weighing from 201 to 350 grams.

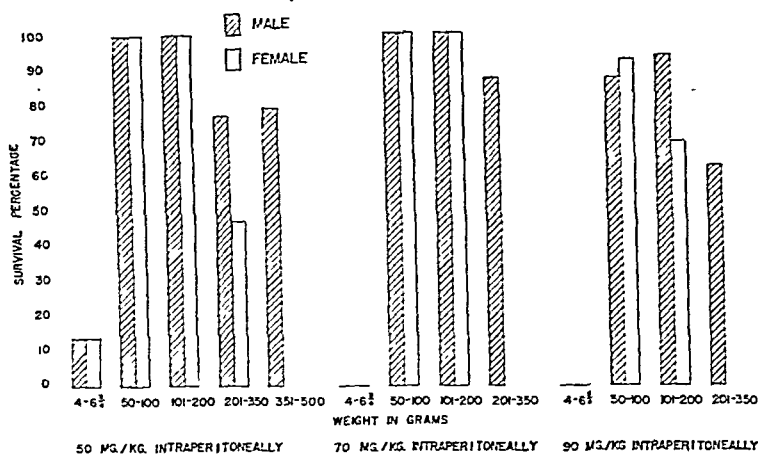


Fig. 2.—Effect of weight and sex on resistance to pentobarbital. Presented in diagrammatic form is the survival percentage, that is, resistance of male and female rats to three different doses of pentobarbital, namely, 50, 70, and 90 mg. per kilogram. The largest of these three doses shows most clearly that the resistance is lowest in the newborn, reaches a maximum sometime while the weight changes from 50 to 100 grams, and then recedes. But even with the lower doses the increasing susceptibility of the aged rats is seen.

Moir<sup>2</sup> observes that in male and female rats weighing about 45 grams each there is a slightly greater resistance in the females. This difference becomes marked when the female rats weigh 55 grams and the male rats 59 grams. This stage of greater female resistance is followed by another in which there is no appreciable difference between the two sexes. This period in which both sexes respond in the same way takes place while the female rats grow from 63 to 81 grams and the male from 68 to 85 grams. After that the relation between the male and female is the opposite of the earlier reaction, for the males become more resistant. The comparatively diminished female resistance is even further impaired when the females weigh 104 grams and the males 113 grams. Thus, the similarity between the reactions of the male and female rats weighing from 50 to 100 grams may be explained by Moir's work as including three periods: (1) greater female resistance, (2) no difference between sexes, and (3) greater male resistance.

It is shown in Table II that a difference in susceptibility is observed even among newborn rats if they are grouped according to weight. The larger and more vigorous newborn rats can tolerate a greater amount of the drug. Yet, with doses of 70 mg. per kilogram or more (results not included in Table II) the mortality is 100 per cent, even with the heaviest members of the newborn group. Irrespective of the differences among the newborn as a group, they are more sensitive to barbiturates than are the adults.



TABLE II. EFFECT OF INTRAPERITONEAL PENTOBARBITAL ON RATS LESS THAN TWENTY-FOUR HOURS OLD

WEIGHT RANGE (GRAMS)	DOSE (30 MG./KG.)	DOSE (40 MG./KG.)	DOSE (50 MG./KG.)
4 to 4 $\frac{1}{4}$	5/7*	6/7	1/1
5 to 5 $\frac{1}{4}$	6/10	19/27	7/7
6 to 6 $\frac{1}{4}$	3/19	4/24	4/6

In order to maintain the same small volume of injection, the concentrations were varied: 30 mg. per kilogram, 0.15 per cent; 40 mg. per kilogram, 0.20 per cent; and 50 mg. per kilogram, 0.25 per cent.

\*Each fraction represents the incidence of mortality; the numerator of fraction indicates the number of animals that died and the denominator the number of animals used.

It is generally accepted that a reciprocal relation exists between metabolic rate and narcotic effect. In the present investigation it has been possible to push this analysis one step further, and it is suggested that this relationship rests for the most part on the metabolic rate of the brain rather than that of the entire body. It is true that in rats the form of the curves for basal metabolic rate<sup>6</sup> and cerebral metabolic rate<sup>7</sup> are similar on a basis of age. Both curves fluctuate within their lowest levels for approximately the first three weeks of life before rising rapidly to a maximum at the end of the seventh week and then receding very slowly toward old age. However, the early changes of basal metabolism rate would seem to depend on the entire organism rather than on the metabolism of any organ, for the metabolism of kidney and liver slices from newborn rats is found to be approximately the same as in the mature animal.<sup>8</sup> Cerebral metabolism, on the contrary, is much lower in the newborn than in the adult,<sup>7</sup> an observation which may explain the lessened resistance of the neonate to barbiturate. This relationship between cerebral metabolism rate and narcosis is to be expected since the primary effect of narcosis is exerted on the brain.

Tables III and IV represent a comparative study of pentothal and compound 897 (N-methyl-pentobarbital).<sup>\*</sup> With strain A, but not with the other strains, the intravenous M.L.D. for a male rat weighing 360 to 460 grams is 50 mg. per kilogram for pentothal and 40 mg. per kilogram for compound 897.

TABLE III. INTRAVENOUS INJECTION OF PENTOTHAL (1 PER CENT SOLUTION)

STRAIN*	SEX	WEIGHT RANGE (GRAMS)	INJECTION TIME IN MINUTES	DOSE MG./KG.	MORTALITY†	EAR REFLEX ABSENT (MINUTES)		SIDE POSITION MAINTAINED (MINUTES)	
						AVERAGE	RANGE	AVERAGE	RANGE
R	M	300 to 420	1 $\frac{1}{2}$	60	3/5	250	141 to 360	375	290 to 460
A	M	360 to 460	1 $\frac{1}{2}$	50	4/7	178	403 to 540	454	403 to 540
R	M	300 to 420	1 $\frac{1}{2}$	50	0/5	127	28 to 360	329	303 to 500
C	M	230 to 250	1 $\frac{1}{2}$	50	0/3	37	32 to 39	254	128 to 420
C	M	260 to 310	$\frac{1}{2}$	30	0/9	16	8 to 28	32	16 to 81
C	F	160 to 200	$\frac{1}{2}$	30	0/9	15	8 to 20	26	15 to 40
R	M	300 to 420	1 $\frac{1}{2}$	25	0/7	19	8 to 30	46	19 to 82
R	M	300 to 420	$\frac{1}{2}$	12.5	0/7	+	+	8 $\frac{1}{2}$	4 to 11
C	M	114 to 150	$\frac{1}{2}$	12.5	0/7	+	+	Not in side position	

\*Strain R came from the Rockland Company; Strain A is that maintained by the Department of Anatomy, Albany Medical College; Strain C came from our own colony in the Department of Physiology and Pharmacology.

†Each fraction represents the incidence of mortality; the numerator indicates the number of animals that died and the denominator the total number of animals used.

\*We are grateful to Dr. R. K. Richards of the Abbott Laboratories, North Chicago, Ill., for supplying this drug.

TABLE IV. INTRAVENOUS INJECTION OF COMPOUND 897 (1 PER CENT SOLUTION)

STRAIN*	SEX	WEIGHT RANGE (GRAMS)	INJECTION TIME IN MINUTES	DOSE MG./KG.	MORTALITY†	EAR REFLEX ABSENT (MINUTES)		SIDE POSITION MAINTAINED (MINUTES)	
						AVERAGE	RANGE	AVERAGE	RANGE
R	M	300 to 420	1½	50	4/5	62	---	120	---
A	M	360 to 460	1½	40	3/7	49	32 to 70	116	99 to 240
C	M	176 to 280	1½	40	0/7	25	17 to 34	48	29 to 88
C	M	260 to 340	½	30	0/7	14	8 to 20	38	22 to 71
C	F	170 to 230	½	30	0/8	45	15 to 72	146	53 to 232
R	M	300 to 420	1½	20	0/7	22	9 to 35	33	23 to 55
R	M	300 to 420	½	10	0/7	÷	÷	8½	7 to 13
C	F	128 to 152	½	10	0/7	÷	÷	6	4 to 8

\*Strain R came from the Rockland Company; Strain A is that maintained by the Department of Anatomy, Albany Medical College; Strain C came from our own colony in the Department of Physiology and Pharmacology.

†Each fraction represents the incidence of mortality; the numerator indicates the number of animals that died and the denominator the total number of animals used.

These results emphasize that differences in the strains of rats and especially in weight (age) are important factors in the responses to the barbiturates. It should also be pointed out that recovery, especially with the higher doses, is much faster with compound 897 than with pentothal.

An interesting difference appears (Table V) between the reactions to a single intravenous injection of the three barbiturates: pentobarbital, pentothal, and compound 897. In these observations the ages of the male and female members of a test group are the same as are the doses in terms of weight. The exact dosage chosen, however, is arbitrary, since 30 mg. per kilogram of pentothal is approximately 60 per cent of its M.L.D. and is 75 per cent of the M.L.D. of compound 897. Nevertheless, the recovery from anesthesia is faster with the latter compound.

Pentobarbital and compound 897 show a difference in the reactions between the two sexes, for the female rats exhibit longer periods of deep and total anesthesia than do the male animals, while with pentothal such a differentiation fails to appear. Similar preliminary observations made on dogs disclose that these animals do not exhibit the sexual differences to compound 897 which are noted in rats. The causes for these characteristic responses of male and female rats to pentobarbital and to compound 897 have not been elucidated, except to the extent that Moir<sup>2</sup> found that the discrepancy diminishes but does not disappear after castration of the male rats. Similarly, the injection of testosterone propionate into female rats cuts down the period of anesthesia significantly.<sup>9</sup>

Data from this laboratory on thiamine-deficient dogs<sup>10</sup> suggest that they exhibit a reduced resistance to pentobarbital, in comparison with animals on a complete diet. Such an impairment of resistance previously has been reported for rats.<sup>11</sup> Pentobarbital becomes more effective not only in animals with thiamine deficiency but also in those with a deficiency of ascorbic acid.<sup>12</sup> We may conclude that in addition to age, sex, weight, strain, and species, diet is also an important factor in determining the responses to barbiturates.

TABLE V. INTRAVENOUS INJECTION OF THREE BARBITURATES (1 PER CENT SOLUTION)

COMPOUND	SEX	AGE IN MONTHS	WEIGHT RANGE (GRAMS)	INJECTION TIME IN MINUTES	DOSE MG./KG.	NUMBER OF ANIMALS INJECTED	NUMBER OF ANIMALS SURVIVED	EAR REFLEX ABSENT (MINUTES)		SIDE POSITION MAINTAINED (MINUTES)	
								AVERAGE	RANGE	AVERAGE	RANGE
Pentobarbital	M	2	170 to 230	$\frac{1}{2}$	40	9	9	62	40 to 86	118	81 to 155
	F	2	130 to 170	$\frac{1}{2}$	40	9	9	100	35 to 130	238	180 to 340
Pentothal	M	3	260 to 310	$\frac{1}{2}$	30	9	9	16	8 to 28	32	16 to 81
	F	3	160 to 200	$\frac{1}{2}$	30	9	9	15	8 to 20	26	15 to 40
Compound 897	M	$3\frac{1}{2}$	260 to 340	$\frac{1}{2}$	30	7	7	14	8 to 20	38	22 to 71
	F	$3\frac{1}{2}$	170 to 230	$\frac{1}{2}$	30	8	8	45	15 to 72	146	53 to 232

## SUMMARY AND CONCLUSIONS

Age, weight, sex, strain within a given species, difference in species, diet, and kind of barbiturate, all influence the reaction to this group of drugs.

1. The newborn rat, not more than twenty-four hours old, is more susceptible than is the adult, while rats weighing 50 to 200 grams are less susceptible than are those weighing 201 to 500 grams. It is possible therefore to form a spectrum of resistance lowest in the newborn, maximum in rats weighing from 50 to 200 grams, and intermediate with advance in weight and age.

2. As growth proceeds the first increase in the greater susceptibility of the female to pentobarbital is observed in rats weighing 101 to 200 grams, for in that weight range the female remains in light anesthesia longer, though the lethality is the same for both sexes. In rats weighing 201 to 500 grams and receiving pentobarbital, not only is the duration of narcosis longer in the female but the lethality is greater.

In response to an intravenous injection both pentobarbital and compound 897 produce more pronounced effects in the female than in the male, but pentothal exhibits no sexual difference.

3. The intravenous M.L.D. for male rats weighing 360 to 460 grams is 50 mg. per kilogram for pentobarbital and 40 mg. per kilogram for compound 897.

A more rapid recovery from anesthesia follows the administration of compound 897 than follows dosage with pentothal.

4. Differences in the reaction to barbiturates are noted in three strains of rats.

## REFERENCES

1. Fitch, R. H., and Tatum, A. L.: Duration of Action of Barbituric Acid Hypnotics as Basis of Classification, *J. Pharmacol. & Exper. Therap.* 44: 325-335, 1932.
2. Moir, W. M.: Influence of Age and Sex on Repeated Administration of Sodium Pentobarbital to Albino Rats, *J. Pharmacol. & Exper. Therap.* 59: 68-85, 1937.
3. Nicholas, J. S., and Barron, D. H.: Use of Sodium Amytal in Production of Anesthesia in the Rat, *J. Pharmacol. & Exper. Therap.* 46: 125-129, 1932.
4. Etsten, B., Alexander, F. A. D., and Himwich, H. E.: Comparative Toxicity of Pentobarbital in Newborn and Adult Rat, *J. LAB. & CLIN. MED.* 28: 706-710, 1943.
5. Carmichael, E. B.: Nembutal Anesthesia; Median Lethal Dose of Nembutal (Pentobarbital Sodium) for Young and Old Rats, *J. Pharmacol. & Exper. Therap.* 62: 284-291, 1938.
6. Kibler, H. H., and Brody, S.: Metabolism and Growth of Rats, *J. Nutrition* 24: 461-468, 1942.
7. Chesler, A., and Himwich, H. E.: Comparative Studies of the Rates of Oxidation and Glycolysis in the Cerebral Cortex and Brain Stem of the Rat, *Am. J. Physiol.* 141: 513-517, 1944.
8. Cutting, M., and McCance, R. A.: The Metabolism of Kidney Slices From New-born and Full-grown Rats, *J. Physiol.* 104: 288-298, 1946.
9. Kinsey, V. E.: Use of Sodium Pentobarbital for Repeated Anesthesia in the White Rat, *J. Am. Pharm. A. (Scient. Ed.)* 29: 387-390, 1940.
10. Himwich, W. A., and Himwich, H. E.: The Influence of Some Organs on the Pyruvate Level in the Blood, *Am. J. Physiol.* 148: 323-326, 1946.
11. Higgins, G. M., and Mann, F. C.: Relation of Vitamin B to Duration of Anesthesia Induced by Pentobarbital Sodium, *S. Clin. North American* 23: 1205-1211, 1943.
12. Richards, R. K., Kueter, K., and Klatt, T. J.: Effect of Vitamin C Deficiency on Action of Different Types of Barbiturates, *Proc. Soc. Exper. Biol. & Med.* 48: 403-409, 1941.

# LABORATORY METHODS

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## A SIMPLIFICATION OF THE SEROLOGIC DIAGNOSIS OF SALMONELLA CULTURES

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RECENTLY, the attention of many workers has been drawn to the Salmonella group by numerous publications on the genus. This increased interest in a group recognized for many years is due to several factors: A rise in enteric infections caused by large movements in population and the makeshift living conditions of the war years, improved methods of isolation of the bacteria, and, finally, the fact that rapid and reliable methods of identification of cultures by serologic analysis are now available.

Faced by a greater demand for accurate diagnosis of enteric infections and his own ability to isolate a larger number of intestinal pathogens by the use of improved selective media, the worker in the average laboratory has been placed at a disadvantage by inability to identify the cultures which are isolated. The multiplicity of Salmonella types and the many sera necessary for their exact identification have prevented workers in small laboratories from taking advantage of the newer knowledge of Salmonella serology. Thus, many laboratories still depend upon agglutination tests in which the O and H reactions of the cultures are not distinguished and the phases of the H antigens are not considered. Such a system inevitably leads to errors in diagnosis which may seriously handicap the work of the epidemiologist.

It is highly desirable that exact methods of identification of the principal pathogens among the Salmonella and approximate methods of diagnosis of the remaining types be made available in simplified form. The public health laboratory should be able to identify with certainty *S. paratyphi A*, *S. paratyphi B*, *S. paratyphi C*, *S. typhi*, and *S. sendai*, since these are strictly human types which produce prolonged febrile diseases, result in a relatively high carrier rate, and tend to become endemic in the population. *S. choleraesuis* also should be identified exactly because it frequently produces septicemia with localizations in man. Finally, it is desirable to identify *S. typhimurium* since it occurs more frequently in man than any other of the numerous types which produce acute gastroenteritis.

Laboratories engaged in the study of diseases of the domestic animals should be able to identify the so-called primary salmonellosis; *S. abortus equi* in horses, *S. dublin* in cattle, *S. abortus ovis* in sheep, *S. choleraesuis* in hogs, and *S. pullorum* and *S. gallinarum* in fowls. Here again it is desirable that

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*S. typhimurium* be identified exactly since it occurs so frequently in all animal species. The remainder of the numerous types which occur in domestic animals may be separated into their respective groups but may not be subjected to exact type determination.

Fortunately, the vast majority of *Salmonella* strains isolated from man and animals are members of groups A, B, C, D, and E of the Kauffmann-White classification. In 1943 Edwards and Bruner<sup>2</sup> found that 98.3 per cent of 3,019 cultures from man and animals were members of these five groups. In 1946 Seligmann, Saphra, and Wassermann<sup>8</sup> obtained an identical figure in 2,916 cultures from man. In 1947 Bruner and Joyce<sup>1</sup> classified 99.5 per cent of 1,007 cultures from man in these groups. Unpublished data of Edwards and Bruner on 12,000 cultures isolated from man and animals indicate that more than 97 per cent of the strains are found in the five previously mentioned groups. Thus, it is evident that the average laboratory need concern itself only with the identification of *Salmonella* cultures which are members of five somatic groups.<sup>2</sup> Other cultures occur so rarely that they may be forwarded immediately to a laboratory which specializes in the study of the genus.

Considering these facts, we propose the following diagnostic scheme for the average routine laboratory. A polyvalent serum which contains agglutinins for antigens I, II, III, IV, V, VI, VII, VIII, IX, X, XII, and XV should be available. This is prepared by injecting a rabbit with a mixture of equal amounts of boiled broth cultures of *S. paratyphi A*, *S. paratyphi B*, *S. thompson*, *S. newport*, *S. gallinarum*, *S. anatum*, and *S. newington*. Details of the preparation of polyvalent sera were published in 1942 by Kauffmann.<sup>7</sup>

Five O sera for groups A to E, respectively, should be prepared as follows:

A	I,II,XII	<i>S. paratyphi A</i>
B	IV,V,XII	<i>S. paratyphi B</i>
C	VI,VII,VIII	<i>S. thompson</i> and <i>S. newport</i>
D	IX,XII	<i>S. gallinarum</i>
E	III,X,XV	<i>S. anatum</i> and <i>S. newington</i>

Methods of preparation of these sera were given in 1941 by Kauffmann<sup>6</sup> and in 1942 by Edwards and Bruner.<sup>2</sup> It will be noted that groups C<sub>1</sub> and C<sub>2</sub> are represented by only one serum as are also groups E<sub>1</sub> and E<sub>2</sub>. In this way the number of necessary sera is reduced.

For the public health laboratory a Vi serum is needed to aid in the identification of *S. typhi* and *S. paratyphi C*. This may be made from a living or an alcohol-treated culture of *S. ballerup* by the method of Edwards and Bruner<sup>2</sup> (1942). Such a serum contains agglutinins for O antigen XXIX and H antigen z<sub>14</sub>, in addition to Vi, but these antigens occur so rarely that the serum may be used without absorption for the determination of Vi antigen.

\*As early as 1930 Kauffmann (Die Technik der Typenbestimmung . . . . . Typhus-Paratyphusgruppe, Zentralbl. f. Bakt. [Abt. 1] 119: 152, 1930) . . . . . set of 12 O-H sera by which the common members of groups A to E could be identified . . . . . 1942 a similar proposal was made by Bornstein (Practical Suggestions for . . . . . determination of *Salmonella* Organisms, J. Bact. 44: 710, 1942.)

The public health laboratory should have six II sera as follows:

a	<i>S. paratyphi A</i>
b	<i>S. huttingfoss</i> , phase 1 (or <i>S. paratyphi B</i> , phase 1)
c	<i>S. choleraesuis</i> , phase 1
d	<i>S. typhi</i>
i	<i>S. aberdeen</i> , phase 1 (or <i>S. bonariensis</i> , phase 1)
1,2,3,5	<i>S. thompson</i> , phase 2, and <i>S. newport</i> , phase 2

Workers in animal pathology should have the c, i, and 1,2,3,5 sera and, in addition, the following:

c,n,x	<i>S. abortus equi</i>
g,p	<i>S. dublin</i>

The preparation of II sera was described in 1941 by Kauffmann<sup>6</sup> and in 1942 Edwards and Bruner.<sup>2</sup>

With this modest array of sera and by use of the ordinary biochemical tests it is possible to identify the principal *Salmonella* types and to group practically all *Salmonella* cultures.

The polyvalent serum is used in slide agglutination tests, usually in a dilution of 1:5 or 1:10, depending upon the titer of the particular serum. It can be used to test colonies directly from plates of selective media or to test growth from slants of differential media to which colonies are transferred. A bit of growth is mixed in saline on a slide and an equal amount of polyvalent sera added or, if desired, the growth may be emulsified directly in the serum dilution. Agglutination becomes apparent after the slide is tilted back and forth for thirty seconds to one minute. In dealing with cultures from man the Vi serum should be used in conjunction with the polyvalent serum so that O-inagglutinable cultures of *S. typhi* will not be missed. If agglutination is not obtained thus with growth from any of the suspicious colonies, those which yield growth resembling *Salmonella* on differential media should be carried through the usual biochemical tests to eliminate the presence of rare *Salmonella* types. If the biochemical tests indicate that the organisms are *Salmonella*, they should be sent to a center for identification.

If agglutination occurs in Vi serum, *S. typhi* should be suspected. In the event that agglutination occurs in the polyvalent serum but in Vi serum, it is probable that a *Salmonella* other than *S. typhi* is present. Cultures which agglutinate in the polyvalent serum should be tested in a similar manner with the sera for the various O groups. While some cross-agglutination occurs between the different O groups, the reactions are usually quite distinct so that no difficulty is encountered in determining to which O group a culture belongs. Cultures which agglutinate in Vi serum should be heated in saline at 100° C. for a few minutes and tested with the O sera. Thus, the *Salmonella* strains can be divided into their respective O groups. Cultures which are initially agglutinable by Vi serum after heating should fall into group C if they are *S. paratyphi C* and into group D if they are *S. typhi*. If no agglutination occurs in the O sera after heating, the heated culture should again be tested in Vi serum.

If agglutination still occurs, it may be concluded that the culture contains XXIX antigen and is not a member of any of the common groups. Very rarely rough cultures of *S. typhi* are isolated which will not agglutinate with group D serum after heating. Thus, it is necessary to determine by biochemical methods whether cultures, which in the living state agglutinate in Vi serum but after heating are inagglutinable by group C, group D, and Vi sera, are in reality rough cultures of *S. typhi*.

After the O group of the culture is determined its H antigens should be examined. This may be done by testing growth from an agar slant on a slide with H sera which are diluted 1:50 or 1:100. An alternate method is that recommended in 1942 by Edwards and Bruner,<sup>3</sup> in which an infusion broth culture is diluted with an equal amount of saline containing 0.6 per cent formalin and tested with appropriate sera at 50° Centigrade. Only one dilution (1:1000) is used and results are read after incubation for one hour in a water bath.

In dealing with cultures from man the group A cultures should be tested with serum a to assure that they are *S. paratyphi A*, the only known type in that group. In addition, they should be subjected to the differential tests, non-fermentation of xylose, and inability to utilize tartrates and citrate. The majority of *S. paratyphi A* strains produce little or no H<sub>2</sub>S.

Group B cultures should be tested with H sera, b, i, and 1,2,3,5. In the event that agglutination occurs in b or i serum, the culture is almost certainly *S. paratyphi B* or *S. typhimurium*, respectively. Diphasic cultures often occur only in one phase when recently isolated. If phase 2 only is present, it is necessary that phase 1 be obtained to identify the culture. Thus, if the culture is agglutinated only by 1,2,3,5 sera, it should be planted on a Gard plate (Kauffmann,<sup>6</sup> 1941) with 1,2,3,5 serum or placed in a tube containing 2 or 3 c.c. of semisolid medium to which a loopful of that serum has been added. Phase 1 will migrate through the medium and can be isolated from the spreading growth. Useful modifications of the Gard technique have been described by Edwards and Bruner<sup>2</sup> (1942), Hajna<sup>4</sup> (1944), and Juenker<sup>5</sup> (1946). After phase 1 is obtained it can be determined whether the culture is *S. paratyphi B*, *S. typhimurium*, or some other type within group B.

Group C cultures should be tested with c and 1,2,3,5 sera. If agglutination occurs in c serum, the culture may be identified as *S. paratyphi C* or *S. choleraesuis* depending upon its biochemical reactions. *S. paratyphi C* usually ferments arabinose and always ferments trehalose promptly whereas *S. choleraesuis* attacks neither of these sugars. *S. paratyphi C* ferments dulcitol promptly while *S. choleraesuis* gives delayed or negative reactions in dulcitol media. If phase 2 only is present, phase 1 should be isolated as previously described and tested with c serum. It should be remembered that the majority of cultures of *S. choleraesuis* belong to the Kunzendorf variety in which phase 1 is more or less completely suppressed, so that any group C culture which agglutinates 1,2,3,5 serum, fails to ferment arabinose and trehalose, and gives a negative or delayed result in dulcitol may be reported as *S. choleraesuis*.



Group D cultures should be tested in a, d, and 1,2,3,5 sera. Cultures which agglutinate only in d serum, which are anaerogenic, and which possess the usual biochemical attributes of *S. typhi* may be reported immediately. The organism will have been examined already for the presence of Vi antigen so that its identity is fairly well established. Some recently isolated cultures of *S. typhi* are very poorly motile and may fail to flocculate in d serum. Such cultures can be typed as *S. typhi* provided they possess IX, XII, and Vi antigens and the biochemical properties of *S. typhi*.

Identification of *S. sendai* depends upon the recognition of both phases and biochemical examination. It is necessary that the organism agglutinate both in a and 1,2,3,5 sera. If only one phase is present the second must be isolated as previously described. Further, this type is H<sub>2</sub>S and citrate negative and gives a negative result in Stern's glycerol-fuchsin broth. Arabinose is fermented immediately but xylose and sorbitol give delayed reactions. Cultures with similar antigens but different biochemical reactions belong to another type. *S. sendai* occurs frequently only in the Orient.

Cultures which belong to group E are not examined further but are recorded simply as group E *Salmonella*.

Cultures of animal origin are treated in much the same manner as human cultures. Group A cultures are known to occur only exceptionally in animals and need not be considered. Group B cultures are tested with c; c,n,x; i and 1,2,3,5 sera. *S. abortus equi* should agglutinate only with c,n,x serum and when planted in semisolid medium containing c,n,x serum it should fail to spread since this type is monophasic. Both phases of *S. abortus ovis* should be recognized. This type should agglutinate in c and 1,2,3,5 sera. Further, the organism should fail to ferment trehalose. It is characterized by a more delicate growth on agar media than that exhibited by most *Salmonella*. *S. typhimurium* is identified as previously described.

Group C cultures are treated as are human strains of group C in order to identify *S. choleraesuis*.

Motile strains of group D are tested with g,p serum to determine whether they are *S. dublin*; *S. enteritidis* also will agglutinate with this serum, but typical Dublin strains can be differentiated from *S. enteritidis* by the fact that *S. dublin* gives a negative or delayed test with arabinose while *S. enteritidis* ferments the sugar promptly.

Nonmotile strains of group D are probably *S. gallinarum* or *S. pullorum*. Such cultures which are anaerogenic and which promptly ferment dulcitol and maltose may be classified as *S. gallinarum*. *S. pullorum* is usually aerogenic but anaerogenic strains occur. Typical cultures ferment neither maltose nor dulcitol although maltose-fermenting strains occasionally are found. Nonmotile cultures which fail to ferment dulcitol may be classified as *S. pullorum*. This type is also characterized by its sparse growth on artificial media.

In summary, it is hoped that through the adoption of a system similar to that previously described, a more satisfactory diagnosis of *Salmonella* types may be achieved in the routine laboratory. Such laboratories should not attempt to type every *Salmonella* culture but should identify exactly only the more

important types. The remaining cultures should be designated "Salmonella, type undetermined" and sent to a center for identification. The adoption of such a system will permit the rapid diagnosis of 98 per cent of Salmonella infections. At present many laboratories still rely on ordinary agglutination tests with sera of doubtful authenticity and specificity and upon biochemical reactions. Reports on Salmonella cultures are therefore often long delayed and inaccurate.

It is realized that this proposal is not strikingly original and that any proposal short of complete typing must be a compromise. However, the system is flexible and can be modified to suit the needs of the individual. The methods proposed here are based upon our experience, with a view toward the employment of a minimal number of sera.

#### REFERENCES

1. Bruner, D. W., and Joyce, B. J.: Salmonella Types Encountered by the 15th Medical General Laboratory, *Am. J. Hyg.* 45: 19-24, 1947.
2. Edwards, P. R., and Bruner, D. W.: The Serological Identification of Salmonella Cultures, *Ky. Agr. Exp. Sta. Circular* 54, 1942.
3. Edwards, P. R., and Bruner, D. W.: The Occurrence and Distribution of Salmonella Types in the United States, *J. Infect. Dis.* 72: 58-67, 1943.
4. Hajna, A. A.: Use of a "U" Tube for the Isolation of Monophasic Varieties From Diphasic Salmonella Cultures, *J. Bact.* 48: 609-610, 1944.
5. Juenker, A. P.: A Rapid Method of Phase Isolation in Salmonella Cultures, *J. Bact.* 52: 609, 1946.
6. Kauffmann, F.: *Die Bakteriologie der Salmonella-Gruppe (Typhus-Paratyphus-Enteritis-Gruppe)*, Copenhagen, 1941, Ejnar Munksgaards Forlag.
7. Kauffmann, F.: Ueber polyvalente Immunseren zur Salmonella-Diagnose, *Acta path. et microbiol. Scandinav.* 19: 248-261, 1942.
8. Seligmann, E., Saphra, I., and Wassermann, M.: Salmonella Infections in the U.S.A., *J. Immunol.* 54: 69-87, 1946.

## THE PROTHROMBIN TIME AND VARIABLES IN THE METHOD

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THE prothrombin test as originally devised by Quick<sup>1</sup> and subsequently modified by Link,<sup>2</sup> Shapiro,<sup>3</sup> Brambel,<sup>4</sup> and others is steadily becoming more important in clinical medicine, particularly as a guide to dicumarol therapy. Accurate, consistent results are imperative if patients are to be adequately treated yet not unnecessarily subjected to the danger of serious hemorrhage. Indeed, if dependable prothrombin values are not obtainable, dicumarol therapy must be discarded.

Our own early experience and observations of some other laboratories lead us to believe that prothrombin results are very often dangerously inaccurate. In the course of our work some factors causing variations have been found. Some have not been mentioned previously and others have received too little emphasis. It is hoped that the recognition of some of the pitfalls may be of value to others and of help in the development of a more reliable technique.

### THE PROTHROMBIN TEST—DESCRIPTION OF TECHNIQUE

All one-stage prothrombin tests used today are based on the original method of Quick. We have followed the Brambel modification of the Quick test because it is more accurate in determining early changes in prothrombin content. This is particularly true between 35 and 100 per cent, where the Brambel dilution curve rises more sharply than do either the dilution curves of Quick or Link and Shapiro (Fig. 1).

Regardless of the method used, each laboratory should determine its own dilution curve in order to report accurately in per cent of prothrombin. Variations in the strength of the thromboplastin and other factors that will be considered later make it impossible to read results from dilution curves that have been made in other laboratories. In the following outline of the method, we follow closely the Brambel technique<sup>4</sup> and note its variations from Quick.

Thromboplastin is prepared from rabbit brain according to the method described by Brambel.<sup>4</sup> It is then kept in a desiccator at 4° C. or sealed in vacuum bottles and kept in the refrigerator. When ready for use, the dried brain containing the thromboplastic substance is removed from the vacuum tubes or the desiccator and ground to a fine powder with a pestle and mortar. (With Quick's method the thromboplastic substance is used without any further grinding.) Then 250 mg. of this powder are suspended in 5.0 c.c. of normal saline solution (0.85 per cent) containing 0.1 c.c. of a 0.1 molar solution of sodium oxalate. After the test tube is thoroughly shaken to insure uniform

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distribution of the particles, it is placed in a water bath maintained at 50° C. for a period of just fifteen minutes. While in the water bath, the test tube is vigorously shaken for ten seconds every two minutes. It is important that the material be shaken regularly and consistently at the two-minute period, as the amount of agitation will change the prothrombin times obtained. Additional shaking will cause times to increase.

At the end of the incubation period the material is put in a centrifuge, and revolved at 500 r.p.m. for just twenty seconds. This will give a milky supernatant fluid which may then be pipetted off and used for testing.

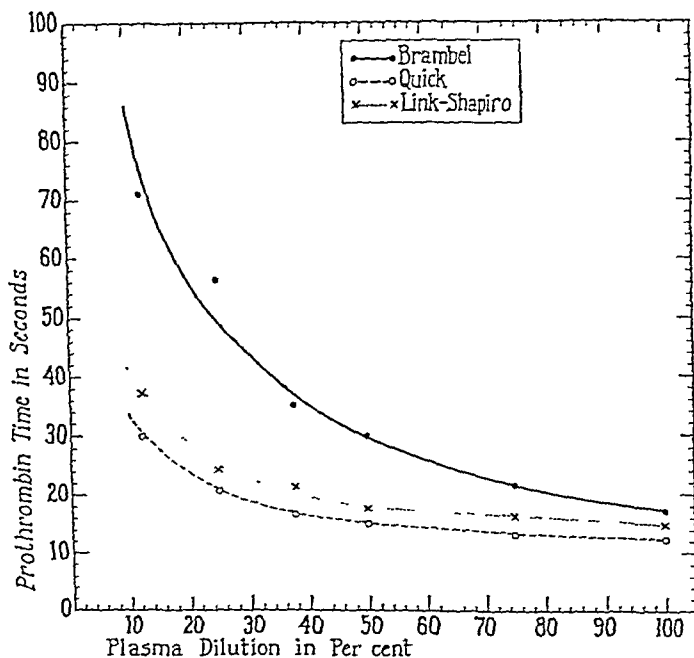


Fig. 1.—Dilution curves obtained by three different methods.

Quick's method of preparation of thromboplastin differs from the previously described method of Brambel only in the following ways: (1) There is no grinding of the acetone-washed, dried brain before adding the normal saline-sodium oxalate mixture; (2) during the period of incubation the only agitation is that produced by the occasional blowing through the mixture with a pipette; and (3) 300 instead of 250 mg. of thromboplastic material are incubated in 50 c.c. of saline-oxalate mixture. This change of amount does not appear to be important, as an excess of thromboplastin is used in both methods.

Plasma for prothrombin determination is obtained by mixing 4.5 c.c. of venous blood with 0.5 c.c. of 0.1 molar sodium oxalate. This is centrifuged at 1,500 r.p.m. for twenty minutes.

One-tenth of a cubic centimeter of plasma is then placed in an 8 by 100 mm. test tube and to this is added 0.10 c.c. of thromboplastin solution. The two are thoroughly mixed and placed in a constant temperature water bath

of 37° C. for two minutes. One-tenth of a cubic centimeter of 0.025 molar calcium chloride, which has previously been allowed to stand at 37° C., is then drawn into a 1.0 c.c. pipette calibrated in 0.10 c.c. graduations to the tip. The calcium chloride is quickly blown into the plasma-prothrombin mixture and at the same moment a stop watch is started. A foot switch for the operation of the watch as described by Shapiro is helpful.<sup>3</sup>

In introducing the calcium chloride into the plasma-prothrombin mixture, it is important to keep several points in mind. The transfer must be made quickly, but care must be taken so that the solutions will not remain on the side of the tube and prevent complete mixing. The pipette used for calcium chloride must be very carefully wiped before it is used again. If this is not done, small drops of the prothrombin-plasma mixture may adhere to the sides of the pipette and contamination of the calcium chloride solution will result, invalidating further tests. A fresh pipette may be used for each transfer of the calcium chloride, but this does not seem necessary if due care is taken.

Once the solutions are mixed by shaking, the mixture is held in the water bath for ten seconds. The tube is then lifted out and gently tilted back and forth until the solution abruptly gels. When coagulation first begins, the stop watch is stopped and the time recorded to the nearest one-tenth of a second. All runs should be done in duplicate and should check within a second for undiluted plasmas.

#### EXAMINATION OF POSSIBLE VARIABLES IN THE METHOD

*Stability of Thromboplastin in Dry State.*—As others have reported, we also have found that the stored thromboplastin loses its potency when exposed to air but that the temperature factor is not so important. According to Quick,<sup>5</sup> when thromboplastin is stored at 4° C. in vacuum containers, potency is maintained over a period of years.

We have tested the same dried thromboplastic substance, prepared according to the Brambel method, over a period of five and one-half months and have found no decrease in the strength. The figures in Table I give the prothrombin times obtained on the same normal individuals over a period of months. In this experiment three separate batches of thromboplastin, kept continuously in a desiccator at 4° C., were used for testing.

TABLE I. STABILITY OF DRIED THROMBOPLASTIN STORED IN DESICCATOR AT 4° C.  
(FIGURES GIVE PROTHROMBIN TIME IN SECONDS)

	DATE TESTED	THROMBOPLASTIN		
		A	B	C
Normal plasma (G. C.)	June 24	17.4		
	July 24		17.2	
	Aug. 19	17.6	17.3	17.4
	Dec. 11	17.2	16.8	17.2
Normal plasma (J. M.)	June 29	17.0		
	July 13		17.1	
	Aug. 16			17.1
	Dec. 11	16.9	16.5	16.8

*Stability of Thromboplastin in Solution.*—It is of some importance to know how long this thromboplastic solution maintains its strength, for time and material may be saved if its stability is such that it may be used for more than one series of tests. In Tables II and III the results of daily tests on the same individuals are shown. The thromboplastic solutions were made up fresh on the first day and kept in the refrigerator at 2° C. for the duration of the experiment. Bloods were drawn at the same time each day on the normal individuals tested, and in all respects the tests were done in the same manner each day.

In Table II are shown the daily times obtained using thromboplastin prepared in our laboratory from the acetone extraction of rabbit brain. When not used for testing, it was kept in the refrigerator. It will be noted that there is a change in potency on the sixth day. This is more noticeable in the first three cases which were done some months before the last four by a different technician and with a different lot of thromboplastin. It would appear justifiable on the basis of these data to use such thromboplastic solutions for at least four days before discarding them.

TABLE II. THROMBOPLASTIN (BRAMBEL), +2° C.  
(PROTHROMBIN TIME IN SECONDS)

CASE	FIRST DAY	SECOND DAY	THIRD DAY	FOURTH DAY	FIFTH DAY	SIXTH DAY	SEVENTH DAY	EIGHTH DAY	NINTH DAY
1	17.2	17.3*	17.4	17.4*	17.4	23.8	24.8		
2	17.1	17.4	17.2	17.5	17.5*	24.8	24.8†		
3	17.2	17.2	17.3	16.0	17.4*	23.9	24.8†		
4	17.7	17.5	17.8	18.4	18.1	21.0	22.0		
5	17.9	18.1	18.2	17.7	19.0	19.1	20.4†		
6	19.0	18.1	18.4	18.0	18.1	18.7	19.1	19.1	19.0
7	20.0	18.9	17.4	17.6	17.7	19.0	21.2	21.2	21.0
Average	18.0	17.8	17.7	17.5	17.9	21.5	22.4		

\*Interpolated.

†Extrapolated.

TABLE III. POTENCY THROMBOPLASTIN (MALTINE) IN SOLUTION, +2° C.  
(PROTHROMBIN TIME IN SECONDS)

NORMAL	FIRST DAY	SECOND DAY	THIRD DAY	FOURTH DAY	FIFTH DAY	SIXTH DAY	SEVENTH DAY	EIGHTH DAY	NINTH DAY
1	16.6	16.0	17.4	18.7	17.6	17.6	19.2	19.1	19.6
2	16.3	16.1	15.9	18.4	18.0	17.4	21.1	18.0	18.0
3	16.7	17.0	16.7	18.3	17.7	17.6	18.9	18.5	21.0
4	16.1	16.6	16.0	18.4	18.0	17.5	19.8	18.8	19.0
5	13.7	16.3	16.5	18.0	17.6	17.5	18.5	18.3	18.1
Average	15.9	16.4	16.5	18.4	17.8	17.5	19.5	18.5	19.1

In many laboratories it is not feasible to prepare thromboplastin from rabbit brain, and the use of a commercial product is necessary. We have therefore extended our observations to one, frequently used, ready-made thromboplastin (Maltine). The experiment was carried out in the same manner as previously described. In this instance there is a significant loss of strength on the fourth day, but values are consistent for the first three days (Table III).

*Calcium Chloride Solution.*—We have used a 0.025 molar concentration of calcium chloride. This is the same strength solution as is used by Shapiro and Brambel. Quick also previously used this solution, but has more recently

changed to a 0.020 molar strength. This may account in part for shorter times observed when using the Quick technique, as the calcium chloride concentration is a critical factor in this test.

Pohle and Stewart<sup>6</sup> showed that even small variations of the calcium chloride had a distinct effect on the prothrombin time. As the calcium chloride strength was reduced to about 0.01 molar the prothrombin times became shorter. Below this level there was a rapid lengthening. We have confirmed these observations, as is shown in Fig. 2. Actually, other molarities than the usual 0.025 molar might be adopted if a dilution curve for that solution were set up and percentage values then derived from that curve. The important consideration is that the calcium chloride solution be consistently of the same strength.

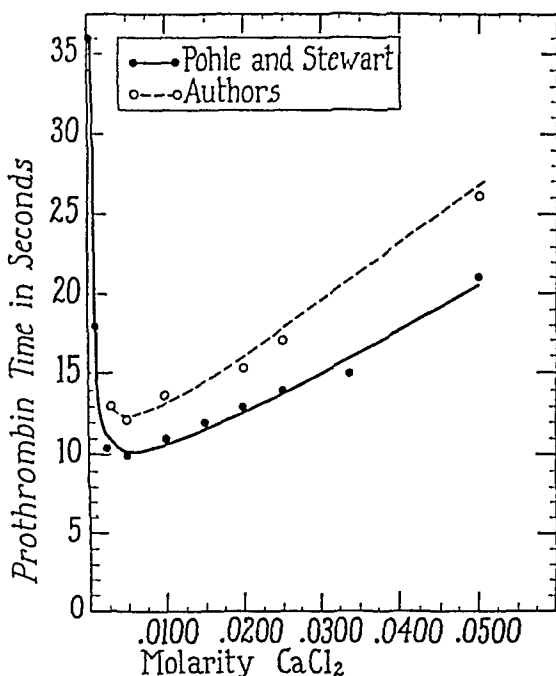


Fig. 2.—Effect of calcium concentration on prothrombin time.

Solutions should be made up accurately and in large enough volume to obviate the necessity of frequent preparation of new stock solutions, which might lead to error. As anhydrous calcium chloride is most hygroscopic, its weight will change even as it is being removed from the container for weighing. For critical work a final chemical analysis of the solution is desirable.

*Plasma—Collection of the Blood.*—Plasma is obtained by mixing 4.5 c.c. of blood with 0.5 c.c. of 0.1 molar sodium oxalate. The mixture is then centrifuged for twenty minutes at 1,500 revolutions per minute. It is important that the vein be entered cleanly and a perfectly dry syringe be used, as slight clotting or hemolysis of the blood will cause shortening of prothrombin times (Figs. 3 and 4).

*Plasma Stability.*—It is usually assumed that the prothrombin test will be run soon after the blood is drawn, but there is little specific information on the stability of the plasma at various temperatures. Link<sup>2</sup> reported that normal rabbit plasma was constant in prothrombin content for several hours at room temperature but that bloods of animals eating spoiled sweet clover (dicumarol equivalent) were likely to change in value after standing three or four hours. Campbell and associates<sup>7</sup> have found that no appreciable error was introduced in keeping plasma obtained from normal rabbits for a week at 0° Centigrade.

In Tables IV to VII tests have been run on both abnormal and normal plasmas, kept for varying times at different temperatures. It will be seen that although there is a more rapid deterioration of prothrombin at high temperatures than at lower ones, prothrombin stability does not vary directly nor consistently with temperature change.

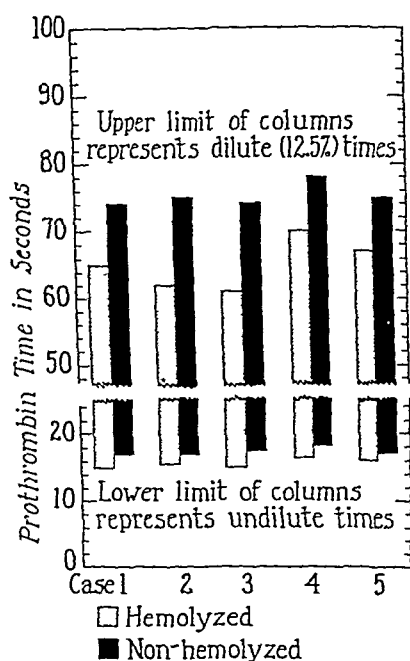


Fig. 3.

Fig. 3.—Effect of hemolysis on prothrombin time.

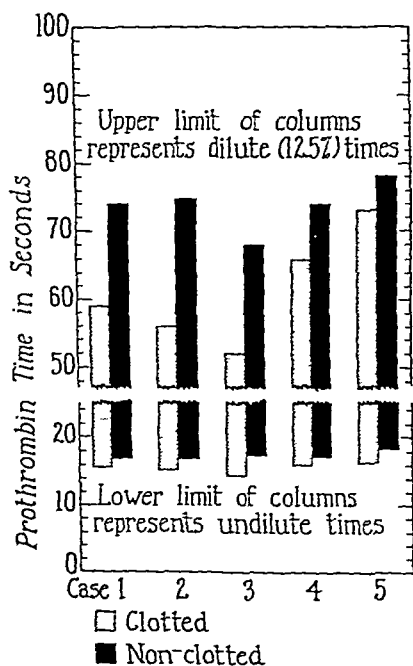


Fig. 4.

Fig. 4.—Effect of clotting on prothrombin time.

In Table IV is shown the loss of prothrombin potency when plasmas are kept refrigerated at 1 to 2° Centigrade. Bloods were drawn at 9:30 A.M., and the plasma was immediately separated by centrifuging and placed in the ice-box. The first determinations were made one hour after bloods were drawn and the second test four hours after obtaining the specimens. There is no significant change up to four hours, but after seven hours the three normals do show definite lengthening of times. At ten hours all show evidence of weakening of the prothrombin strength. It is obviously impossible to keep plasmas overnight in a refrigerator and still obtain accurate results.



TABLE IV. PLASMA STABILITY, 1 TO 2° C.  
(PROTHROMBIN TIME IN SECONDS)

CASE	TIME AFTER DRAWING OF BLOOD			
	1 HOUR	4 HOURS	7 HOURS	10 HOURS
1	18.5	17.8	17.8	20.5
2	29.8	27.0	29.0	33.0
3	33.0	33.1	32.4	35.3
4	29.9	31.8	32.3	37.2
5	39.7	37.0	40.0	44.1
6*	15.5	15.5	17.8	19.5
7*	14.8	15.6	17.1	17.8
8*	15.1	15.2	17.7	18.2

\*Normals.

TABLE V. PLASMA STABILITY, 21° C. (70° F.)  
(PROTHROMBIN TIME IN SECONDS)

CASE	TIME AFTER DRAWING OF BLOOD				
	1 HOUR	2.5 HOURS	4 HOURS	5 HOURS	6 HOURS
1*	15.8	15.7	15.5	16.1	16.1
2*	15.8	15.9	15.7	15.9	16.2
3	24.3	23.9	23.2	24.8	26.3
4	18.7	18.8	19.6	20.9	22.0
5	20.0	28.7	29.8	31.6	32.6
6	28.0	27.4	27.1	27.7	27.4
7	27.3	27.4	26.8	28.6	31.1
8	30.2	30.1	31.9	32.2	34.9

\*Normals.

In a similar manner blood samples were obtained and the plasmas allowed to remain at a room temperature of 21° C. (70° F.). Loss of potency was not greatly accelerated as compared to plasmas kept in the icebox, but in five hours' time some samples gave evidence of slight loss of prothrombin activity. In six hours five of the eight gave longer values (Table V).

A series run during the summer months at a room temperature of 29° C. (89° F.) showed no significant variation at five and one-half hours but considerable deterioration of prothrombin activity after six and one-half hours (Table VI). It appears, therefore, that with the room temperatures usually

TABLE VI. PLASMA STABILITY, 29 TO 31° C. (84 TO 88° F.)  
(PROTHROMBIN TIME IN SECONDS)

CASE	TIME AFTER DRAWING OF BLOOD							
	1.5 HOURS	3.5 HOURS	4.5 HOURS	5.5 HOURS	6.5 HOURS	7.75 HOURS	9.25 HOURS	10.25 HOURS
1*	17.0	16.9	17.7	16.5	21.6	26.8	21.9	26.1
2*	45.4	45.7	46.8	43.8	58.0	63.0	53.0	61.0
3	26.6	25.0	27.9	24.0	32.6	33.7	32.5	39.1
4	17.7	17.6	18.0	17.6	22.7	26.4	28.0	24.7

\*Normals.

TABLE VII  
(PROTHROMBIN TIME IN SECONDS)

CASE	45 MINUTES AFTER DRAWING BLOOD (TEMPERATURE 21° C.)	1 HOUR LATER (PLASMA KEPT AT 37° C.)
1*	14.8	18.9
2*	15.1	18.3
3	18.5	22.8
4	29.8	36.1
5	37.9	43.7

\*Normals.

encountered (70 to 84° F.) there is little variation in rate at which prothrombin activity is lost.

All specimens should be run as soon as possible after the bloods are drawn, and any plasma kept longer than five hours either at room or icebox temperatures cannot be interpreted with accuracy.

When the temperature is raised to 37° C., loss of potency is much more rapid, as is shown in Table VII. This sudden rise in rate of prothrombin destruction is somewhat surprising in view of results noted at lower temperatures. We are unable to explain this variation in action. Perhaps prothrombin loss is due to the action of enzymes present in the plasma or it may be a result of bacterial activity. No attempt has been made to run these tests under strict aseptic conditions.

*Plasma—Time of Taking Blood Samples.*—Meyers and Poindexter,<sup>8</sup> using the dilute prothrombin method of Shapiro, showed that patients with coronary arteriosclerosis had a slightly shorter prothrombin time in the evening and early morning hours. Levan<sup>9</sup> states that "determinations after breakfast were considerably higher in per cent prothrombin than fasting specimens." That is, the prothrombin times after breakfast were shorter.

We have run serial determinations throughout the day on ambulatory patients. Since other work being carried on in the laboratory at this time necessitated the use of the Link-Shapiro method, it was employed in these particular determinations.

The first specimens were taken at 8:30 A.M. and breakfast was eaten immediately. A second blood was drawn at 10:30 A.M., approximately two hours after breakfast. Lunch was eaten at 11:45 A.M., and a midafternoon sample was taken at 3:00 P.M. Supper was served at 5:00 P.M., and the fourth and last blood was drawn at 11:00 P.M. We did not find any significant difference between bloods drawn before and after breakfast, and it would not appear necessary to insist on fasting blood samples. There is, however, consistent lengthening of the prothrombin times of the 3:00 P.M. samples when these are compared to morning samples. The specimens drawn at 11:00 P.M. have somewhat shorter times than those in midafternoon but are longer than morning tests (Table VIII).

TABLE VIII. DIURNAL VARIATION (MALTINE THROMBOPLASTIN) (TIME IN SECONDS)

CASE	8:30 A.M.	10:30 A.M.	3:00 P.M.	11:00 P.M.
1	14.5	15.0	15.5	15.1
2	15.5	16.0	16.5	16.2
3	15.3	15.2	16.2	15.3
4	15.2	15.2	16.8	15.9
5	15.6	15.6	18.0	17.1
6	15.2	16.2	17.2	16.2
Average	15.2	15.5	16.7	16.0

Meals at: 8:40 A.M., 11:45 A.M., and 5:00 P.M.

For maximum accuracy it is advisable to take blood samples at the same time each day. It may well be that patients on dicumarol show even wider diurnal fluctuations, but this is impossible to demonstrate, as any such change would be obscured by fluctuations in levels due to the action of the drug itself.

TABLE IX

TYPE OF MEAL	TIME OF SPECIMEN	PROTHROMBIN TIME (SECONDS)		
		PATIENT 1	PATIENT 2	PATIENT 3
Breakfast with 20 Gm. fat	Fasting	17.5	17.0	17.4
	Two hours after meal	14.7*	16.9	16.9
Breakfast with 50 Gm. fat	Fasting	17.6	17.8	17.5
	Two hours after meal	17.1	17.4	16.9

\*Hemolyzed blood.

It may be that the degree of lipemia of the blood following meals may account for a slight diurnal variation. Pohle and Stewart,<sup>6</sup> using the Quick method, found a shortening of times after a meal heavy with fat. We have not been able to show any difference in the undiluted times on bloods taken (1) two hours after an average breakfast containing 20 to 30 Gm. of fat and (2) those taken before and two hours after a breakfast containing 50 Gm. of fat (Table IX).

*Variations in the Running of the Prothrombin Test (Quick Method or Brambel Modification).—*

*Water Bath Temperature:* As shown in Tables X and XI, no great error is introduced if the temperature of the water bath is changed from 35 to 40° Centigrade.

TABLE X. EFFECT OF TEMPERATURE CHANGE OF WATER BATH ON PROTHROMBIN TIMES (BRAMBEL TECHNIQUE AND THROMBOPLASTIN PREPARED IN OUR LABORATORY) (TIME IN SECONDS)

TEMPERATURE	UNDILUTED PLASMA				12.5% SALINE DILUTION			
	CASE				CASE			
	1	2	3	4	1	2	3	4
35° C.	16.5	17.0	26.8	31.4	67.0	78.0	177	307
37.5° C.	16.6	17.0	27.0	31.2	66.0	79.0	181.5	310
40° C.	15.5	16.9	25.7	30.5	66.5	71.3	181.5	306

TABLE XI. EFFECT OF TEMPERATURE CHANGE IN WATER BATH (LINK-SHAPIRO TECHNIQUE WITH MALTINE THROMBOPLASTIN) (TIME IN SECONDS)

TEMPERATURE	UNDILUTED PLASMA				12.5% SALINE DILUTION			
	CASE				CASE			
	1	2	3	4	1	2	3	4
35° C.	14.3	14.9	23.7	26.2	32.8	34.8	55.0	64.5
37.5° C.	14.4	15.0	23.3	25.5	31.9	35.3	58.5	62.8
40° C.	14.0	14.5	21.8	25.0	31.6	34.1	54.5	65.0

TABLE XII  
(PROTHROMBIN TIME IN SECONDS)

CASE	TILT TUBE METHOD	WIRE LOOP METHOD
1	21.9	21.6
2	15.7	16.1
3	24.9	24.6
4	15.9	16.7
5	24.5	24.9
6	27.2	26.8
7	15.3	15.2
8	15.0	14.5
9	16.1	15.8
10	25.2	24.4

The same lack of variation in times with changes in temperature of the water bath is also found when the Link-Shapiro method is used with maltine thromboplastin.

*Use of Tilt Tube or Wire Loop Method:* Some workers determine the time of clotting by passing a thin wire loop through the solution and noting when the fibrin first adheres to the wire. We have run parallel determinations on the same plasmas using this wire method and the tilt-tube technique. No consistent difference is apparent in the results shown in Table XII.

*Order of Mixing Reagents:* There is another variation in the running of the test that does alter the results. In the Link-Shapiro method the calcium chloride and thromboplastin are first mixed together and finally 0.10 c.c. of plasma is added just as the stop watch is started. In the Quick and Brambel methods the thromboplastin and plasma are first mixed and the calcium chloride is added last. We have found that this minor change in technique will give a consistent difference in results. When the calcium chloride is first mixed with thromboplastin and the plasma is added last, the times are shorter than when there is premixing of plasma and thromboplastin with a final addition of calcium chloride. The results of the two methods run on twelve consecutive plasmas are shown in Fig. 5. Both methods give reproducible times, but it is apparent that the same technique must be consistently followed.

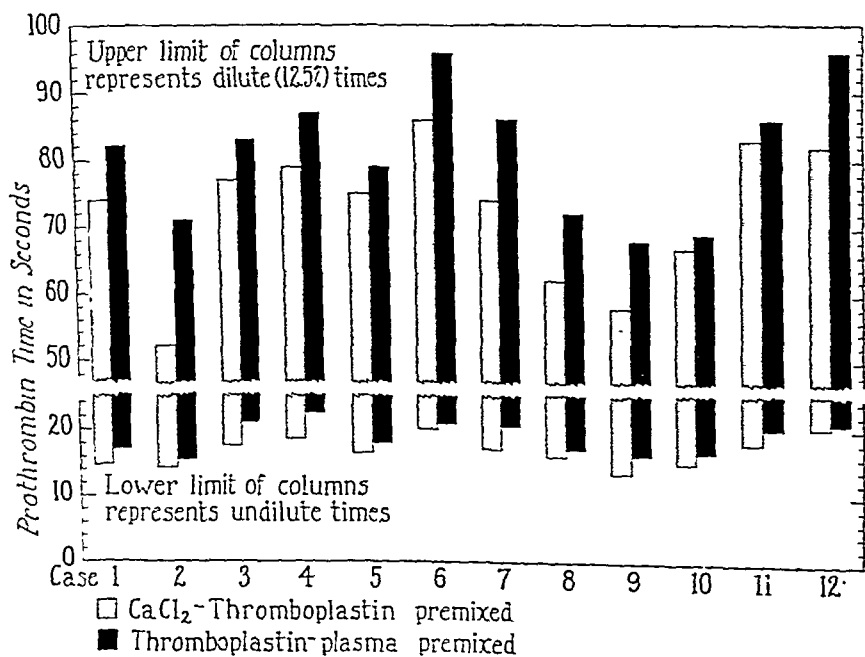


Fig. 5.—Effect of changing the order of mixing the reagents.

#### RANGE OF NORMAL

Abnormal findings cannot be properly evaluated until the range of normal is known. Too few data have been published on normal prothrombin times as

TABLE XIII. REPEATED DETERMINATIONS ON FOUR NORMAL MEN AND WOMEN

CASE	SEX	NUMBER OF DETERMINATIONS	UNDILUTE			DILUTE		
			MEAN	S.D.	(RANGE)	MEAN	(S.D.)	RANGE
1	M	9	17.1	± .3	16.6 to 17.6	77	±7	69 to 101
2	M	11	17.3	± .7	16.1 to 18.6	91	±8	74 to 120
3	F	10	17.0	± .5	15.8 to 17.5	71	±2	67 to 74
4	F	10	17.1	±0.5	16.0 to 17.5	75	±8	66 to 96

measured by the various methods, and care must be taken lest times be reported as "abnormal" when they really come within normal range.

The figures in Table XIII will serve to show the variations possible in this method in a group of healthy individuals. Tests were done on two normal men and two normal women at various intervals over a period of several weeks. The maximum range in the undiluted test was 2.5 seconds and in the 12.5 per cent saline dilution 46 seconds.

In Table XIV are shown the variations in times obtained in a group of ninety-three normal people, fifty-eight women and thirty-five men. Most of these were nurses and medical students between the ages of 20 and 30 years of age. Three different batches of thromboplastin were used. It will be noted that while the standard deviation for the undilute plasma is only 0.6 seconds, the range of normal is three seconds. Dilute times are much less consistent, with a standard deviation of ten seconds and a range of normal from 56 to 115 seconds. Therefore, moderate variations in the dilute times, as obtained by this method, are of doubtful significance. Work now in progress leads us to believe that the practice of diluting the plasma to 12.5 per cent with saline, as is advocated by those using the "dilute method," leads to the introduction of uncontrolled variables and gives inaccurate results. As a matter of fact, the use of saline in making a dilution curve is probably not entirely sound.

TABLE XIV. RANGE OF NORMAL IN HEALTHY INDIVIDUALS  
(NINETY-THREE NORMAL INDIVIDUALS)

BATCH OF THROMBOPLASTIN	MEN	WOMEN	TOTAL	MEAN	UNDILUTE (S.D.)	RANGE	MEAN	DILUTE (S.D.)	RANGE
A	5	12	17	17.2	±.7	15.4 to 18.0	83	±17	61 to 115
B	2	9	11	17.4	±.6	16.9 to 18.3	74	±12	63 to 96
C	51	14	65	17.2	±.6	16.3 to 18.4	74	± 8	56 to 102
Over-all total	58	35	93	17.2	±.6	15.4 to 18.4	76	±10	56 to 115

## SUMMARY

1. The prothrombin test as devised by Quick and modified by Brambel has been reviewed in detail and the effect of certain variables examined. These include observations on:

- The stability of thromboplastin in solution and the dry state
- The effect of changing the molarity of the  $\text{CaCl}_2$  solution
- The effect of hemolysis and clotting of the blood samples
- The effect of various temperatures on the plasma prothrombin stability
- The effect of changing the order of mixing the reagents

- (f) The effect of meals and time of day on resulting times
- (g) The effect of water bath temperature in running the test

2. The bloods of ninety-three normal persons were tested for prothrombin content. According to the method described, the following values were obtained:

(a) Mean time for undilute plasma was 17.2 seconds with a standard deviation of 0.6 seconds and a range of from 15.4 to 18.4 seconds.

(b) Mean time for 12.5 per cent saline dilution of plasma was 76 seconds with a standard deviation of 10 and a range of 56 to 115 seconds.

3. Repeated tests were made on the same normals and mean standard deviation and range were computed for dilute and undilute specimens.

The authors wish to express their appreciation to Dr. Joseph M. Hayman, Jr., for his many helpful suggestions in the preparation of the manuscript.

#### REFERENCES

1. Quick, A. J.: *The Hemorrhagic Diseases and the Physiology of Hemostasis*, Springfield, Ill., 1942, Charles C Thomas, Publisher.
2. Link, K. P.: *The Anticoagulant From Spoiled Sweet Clover Hay*, *Harvey Lect.* 39: 162-216, 1944.
3. Shapiro, S.: Hyperprothrombinemia, a Premonitory Sign of Thromboembolization (Description of a Method), *Exper. Med. & Surg.* 2: 103-109, 1944.
4. Brambel, C. E.: Thromboplastic Reagent, *Arch. Surg.* 50: 137-147, 1945.
5. Quick, A. J.: On the Quantitative Estimation of Prothrombin, *Am. J. Clin. Path.* 15: 560-566, 1945.
6. Pohle, F. J., and Stewart, J. K.: A Study of the Quick Method for the Quantitative Determination of Prothrombin With Suggested Modifications, *Am. J. M. Sc.* 198: 622-630, 1939.
7. Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P.: Studies on the Hemorrhagic Sweet Clover Disease, *J. Biol. Chem.* 138: 1-33, 1941.
8. Meyers, L., and Poindexter, C. A.: A Study of the Prothrombin Time in Normal Subjects and in Patients With Arteriosclerosis, *Am. Heart J.* 31: 27-32, 1946.
9. Levan, J. B.: Dicumarol Therapy, *Ann. Int. Med.* 25: 941-949, 1946.

# A METHOD FOR COUNTING BACTERIA IN THE NASAL CAVITY

## APPLICATION OF THE METHOD IN DEMONSTRATING THE ACTION OF INTRANASAL PENICILLIN ADMINISTRATION

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### INTRODUCTION

THE usual method of studying the types of bacteria in the nasal cavity is to insert a sterile cotton swab and culture it in liquid or solid media. Such a procedure is satisfactory for a qualitative examination of the nasal flora, as in the diagnosis of diphtheria or other nasal infections, but is totally inadequate for an estimation of the number of bacteria present.

The reasons for the inadequacy of this method for quantitative purposes become readily apparent from the following points of discussion. It is known (Thomson and Thomson<sup>1</sup>), and we have confirmed the fact, that the vestibule of the nostril is colonized with a great number of bacteria, usually air-borne saprophytes, which may contaminate the swab as it is inserted or withdrawn and thus distort the true picture of the types and number of bacteria found within the nasal cavity. The sine qua non of making a quantitative estimate of the number of bacteria from a given liquid or surface at repeated intervals for comparative purposes is the use of a precisely uniform sampling method; this appears to be virtually impossible when inserting a swab into the nasal cavity at repeated intervals. Abnormalities in the topography of the nasal cavity often render many areas inaccessible to the swab. It is entirely possible that nasal bacteria are not uniformly distributed over portions of the cavity which are accessible to the swab. Differences in pressure when inserting the swab may also account for variations in the number of bacteria that are recovered on the swab. All of the bacteria removed by the swab may not be dislodged when it is cultured, especially on a solid medium. These few considerations indicate some of the probable causes why the swab method, which we have tested extensively, is unsatisfactory for obtaining a reliable count of the number of bacteria in the nasal cavity.

Recently, Hamburger and co-workers<sup>2, 3</sup> have devised novel methods for obtaining an estimate of the number of streptococci in the nasal cavity. These consist either of having the subject blow his nose on a sterile cloth which is subsequently cultured in toto and the number of streptococci determined by plate counts of the sample or of pinning a cloth patch to the bed in which the subject sleeps and determining the number of streptococci adhering to the cloth. While both of these methods gave useful information in these workers' experiments, they were admittedly crude and the results were sub-

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ject to considerable variation. So far as we are aware, no other method has been published which gives more reliable knowledge of the number of bacteria in the nasal cavity than those previously mentioned.

In determining the efficacy of intranasally instilled antibacterial agents, it is obviously necessary to employ a method of sampling the nasal flora which will give reasonably reliable and consistent results. For this purpose a simple sampling device and procedure have been developed which are believed to give more reliable quantitative results in obtaining a count of the nasal bacteria than any other procedure thus far recorded in the literature. A description of this sampling device, recommended procedure in sampling, and results obtained are reported in this paper.

#### SAMPLING DEVICE AND PROCEDURE

The construction of the sampling device which can be readily prepared in most laboratories is shown in Fig. 1. It consists of a test tube 13 by 2.5 cm. with the closed end drawn out to a diameter of 0.5 cm. and cut off. This end of the tube is connected to a glass nasal "olive" by a piece of rubber tubing 20 cm. in length. The opening in the nasal olive measures 1 cm. in diameter at the end inserted into the nostril, 2 cm. at the bulge, and 0.5 cm. at the end connected to the rubber tubing. Variations in these dimensions can be made, of course, but we have found this size satisfactory. The open end of the test tube is plugged with cotton, the nasal olive wrapped in paper, and the entire device sterilized in the autoclave.

In using the sampling device, 20 c.c. of sterile broth or Ringer's solution (the latter being preferred since it is less objectionable to most subjects) are pipetted aseptically into the test tube and the cotton plug replaced. The paper is removed from the olive which is inserted tightly into one nostril with one hand while the test tube is held with the other hand. The head is bent slightly forward and the subject slowly raises the tube, as shown in Fig. 2, until he feels the solution just beginning to trickle down into the throat. At this point he lowers the tube which siphons the solution back into the test tube. This process is repeated two times to insure a thorough washing of the nasal cavity.

The nasal washings thus obtained are run through the nasal olive into a sterile, empty, rubber-stoppered 100 c.c. dilution bottle (French square type) which is shaken twenty-five times to disintegrate clumps of organisms. One cubic centimeter of this suspension as well as 1 c.c. of 1:10 and 1:100 dilutions are plated in blood agar to determine the total number of bacteria per cubic centimeter of the nasal washings. Many variations of this sampling procedure suggest themselves, and we have tested the following: (1) Making five successive washings at five-minute intervals with fresh solutions each time and plating each washing individually; (2) making three successive washings and combining the washings before plating; (3) washing both nostrils and combining the washings; and (4) making washings in the morning and afternoon or only once in the same day.



As a result of these preliminary trials, the following procedure was chosen finally as giving the most reliable results for testing the antibacterial action of intranasally instilled agents. In order to determine the average daily normal count for control purposes, at the same time each day for a week or more preceding the use of the agent, a sample is obtained from each subject as follows: The nasal olive is inserted into one nostril and the irrigating solution allowed to flow in and out of the nasal cavity twice; then the olive is inserted into the other nostril and the process repeated. Two additional sampling devices, each filled with 20 c.c. of solution, are used successively in the same way. All three washings (60 c.c.) are then pooled, shaken, and cultured as described previously.

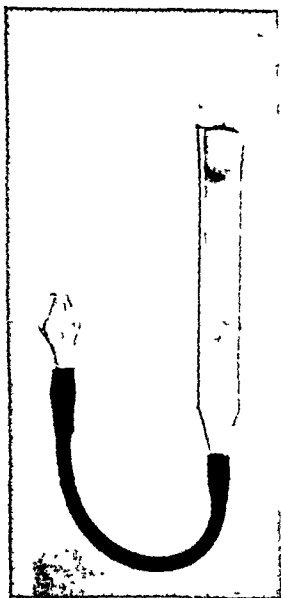


Fig. 1.



Fig. 2.

Fig. 1.—Sampling device for irrigating the nasal cavity.

Fig. 2.—Showing the position of the sampling device for irrigation of the nasal cavity.

During the following week the antibacterial agent is applied in accordance with the requirements of the experiment and nasal washings are carried out at the same time of day as the preceding week. The next week no intranasal applications are made and washings are continued at the usual time.

#### RESULTS

To illustrate the degree of variation which may be expected when daily counts are made on healthy subjects without the application of an effective intranasal agent, the following protocol is typical of many of our experiments (Table I).

It will be noted that while there are wide daily variations in the counts, within certain limits the subjects tend to remain in high (Subjects C

TABLE I. NASAL BACTERIA COUNTS ON HEALTHY SUBJECTS; NUMBER OF BACTERIA PER CUBIC CENTIMETER OF NASAL WASHINGS

DATE	SUBJECT				
	A	B	C	D	E
4/29	360	900	9,920	90	26,220
4/30	800	2,000	1,200	330	18,000
5/1	70	860	1,700	400	13,500
5/2	90	260	200	250	10,700
5/3	90	320	2,200	160	1,000
5/6	1,480	520	5,100	400	12,200
5/7	490	630	13,000	110	17,500
5/8	220	1,300	200	120	38,200
5/9	840	1,330	3,000	100	19,800
5/10	530	500	—	300	2,800
Average	497	862	4,058	225	15,992

—, Subject not available.

TABLE II. NASAL BACTERIA COUNTS ON HEALTHY SUBJECTS BEFORE, DURING, AND AFTER PENICILLIN ADMINISTRATION; NUMBER OF BACTERIA PER CUBIC CENTIMETER OF NASAL WASHINGS

DATE	SUBJECT				
	A	B	E	F	G
Control period					
3/5	1,200	590	52,100	185	15,000
3/6	560	310	—	172	3,600
3/7	128	400	46,000	360	1,100
3/8	236	720	35,000	260	600
Average	531	505	44,366	244	5,075
Penicillin administration					
3/11	52	80	240	12	96
3/12	6	4	350	5	18
3/13	0	0	324	1	12
3/14	0	0	250	0	8
3/15	3	2	195	3	—
Average	12	17	271	4	33
Control period					
3/18	3	26	—	1	47
3/19	0	48	—	2	—
3/20	0	210	—	30	480
3/21	2	65	—	22	68
3/22	18	400	—	—	1,128
Average	4	149	—	14	430

—, Subject not available.

and E), medium (Subjects A and B), or low (Subject D) count groups. Because of this great variation in normal counts, an intranasally applied antibacterial agent must show a very marked reduction in the counts before its effectiveness can be proven statistically. That such a reduction can be achieved is shown by the results in Table II, when the subjects received nose drops containing 500 units of penicillin per cubic centimeter six times daily at hourly intervals and nasal washings were carried out forty to sixty minutes after the last application of penicillin. The washings from three subjects were seeded with a known concentration of penicillin-susceptible *Staphylococcus aureus* organisms and plated in the usual way. These plates did not show any reduction in counts when compared with the control *Staph. aureus* cultures; hence, it is concluded that a carry over of significant amounts of penicillin into the plates did not occur.

There appears to be no doubt that the application of penicillin nose drops produced a marked reduction in the number of nasal bacteria recovered from healthy subjects. In addition, the repressive effect of penicillin seems to persist for a number of days after use. It is interesting to note that Subject E failed to show as low a total count during the penicillin applications as the other subjects. The fact that most of the organisms cultured during this period from the subject were gram-negative, nonsporulating bacilli probably explains this finding.

#### DISCUSSION

Since the number of bacteria in the nasal cavity probably fluctuates from day to day, the ideal procedure would be to make a control count in the morning, apply the antibacterial agent throughout the day, and make a second count in the late afternoon or evening. However, when this was tried it was found that the morning irrigation alone produced such a marked reduction in the afternoon counts that it was difficult to interpret the effectiveness of an antibacterial agent tested by this procedure. Hence, it appeared necessary to make nasal counts for a week or more before the experiment to obtain an average normal count; then apply the antibacterial agent and compare the counts obtained. As a further check, counts should be made for a week or longer after use of the antibacterial agent is discontinued; if the counts tend to return to the normal level after a reduction during use of the antibacterial agent, it is a strong indication that the agent has been effective. No experiment should be considered satisfactory unless it can be shown that the antibacterial agent is not carried over in the washings in sufficient concentration to exert a bacteriostatic action in the culture plates.

During the trials with five successive washings it was discovered that the first washing removed very few bacteria, while the successive washings removed increasingly more bacteria until a point was reached where further washings showed a progressively diminishing count. Thus, it is essential that each sample consist of at least three separate washings if the most reliable count is to be obtained.

It is believed that the method described in this report for obtaining a quantitative count of the number of bacteria in the nasal cavity offers a reasonably reliable tool in determining the antibacterial action of intranasal agents. It is obvious also that this method may be used for a qualitative as well as a quantitative study of the nasal flora; that is, a differential count of the various types of bacteria may be made when the blood agar plates are examined. The procedure does not pretend to count all of the bacteria in the nasal cavity, since the irrigating solution obviously does not reach all portions of the cavity, but it appears to produce a representative sample of the flora, by washing out approximately the same area of the cavity on repeated irrigations. Variations in counts may be expected due to leakage around the nasal olive and spilling over into the throat, but in our experience subjects soon learn to avoid these losses after a little practice and the nasal counts become correspondingly more reliable.

Finally, it should be emphasized that the reduction in nasal bacteria of healthy subjects by penicillin reported in this paper does not constitute a direct or implied indication that penicillin is of value in the prevention, treatment, or cure of nasal or other respiratory infections.

#### SUMMARY

A simple sampling device for washing the nasal cavity with saline or broth is described.

A recommended procedure for using the sampling device to determine the number and types of nasal bacteria is outlined.

The effectiveness of penicillin nose drops in reducing the number of nasal bacteria in healthy subjects, as indicated by the results obtained with the sampling device, is described.

#### REFERENCES

1. Thomson, D., and Thomson, R.: The Common Cold With Special Reference to the Part Played by Streptococci, Pneumococci, and Other Organisms, *Ann. Pickett-Thomson Research Lab.* 8: 1-699, 1932.
2. Hamburger, M., Jr., Green, M. J., and Hamburger, V. G.: The problem of the "Dangerous Carrier" of Hemolytic Streptococci. I. Number of Hemolytic Streptococci Expelled by Carriers With Positive and Negative Nose Cultures, *J. Infect. Dis.* 77: 68-81, 1945.
3. Hamburger, M., and Lemon, H. M.: The Problem of the Dangerous Carrier of Hemolytic Streptococci. III. The Chemotherapeutic Control of Nasal Carriers, *J. A. M. A.* 130: 836-841, 1946.

# A MODIFIED TECHNIQUE FOR THE DIFFERENTIATION OF PNEUMOCOCCI FROM ALPHA HEMOLYTIC STREPTOCOCCI USING SODIUM DESOXYCHOLATE

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WITH THE TECHNICAL ASSISTANCE OF KATHERINE P. SIMPSON, B.S.

THE differentiation of the pneumococcus from the alpha hemolytic streptococcus often is impossible on a purely morphologic basis. At present, the chief means of differentiating these two organisms are the time-honored bile solubility test and the results obtained from typing with Neufeld type-specific sera. Today, this latter method often is time consuming because so many patients have had sulfonamide therapy by the time they enter the hospital. Thus, the suspect pneumococci in sputum often will not type unless passed through one or two mice in order to relieve the organisms of the sulfonamide effects. Therefore, in the large percentage of cases, the bile solubility test has offered the most practical means of differentiation.

Since Neufeld's<sup>1</sup> discovery in 1900 that the pneumococcus was soluble in whole bile, many investigators have described various substances and numerous methods by which this organism may be dissolved. By 1907 the pneumococcus was first distinguished by its bile solubility; it was at this time that Nicolle and Adil-Bey<sup>2</sup> recognized that bile salts (sodium cholate and sodium choleate) were more effective than whole bile. Though Dochez and Gillespie<sup>3</sup> apparently found a true bile-insoluble strain of pneumococcus, according to White and associates<sup>4</sup> Mair states: "Different strains of pneumococcus show varying sensitiveness to the action of bile, just as they vary in the readiness with which they undergo autolysis in culture; but with a satisfactory technique one is seldom in doubt as to whether a particular strain should be classed as bile soluble or not, and strains which have been kept on culture media for long periods retain the property." A thorough review of the literature pertinent to this subject is given by White and associates.<sup>4</sup>

Liefson's<sup>5</sup> modification of the bile solubility test proved unsuccessful in our hands. The purpose of this paper is to present what we believe to be a faster and simpler test, which has proved to be accurate. This test, requiring little equipment, is suitable for the laboratory of the intern or for the general hospital.

## METHOD

*Equipment.*—The equipment, as illustrated in Fig. 1, includes the following:

1. Fresh solution of 10 per cent sodium desoxycholate, pH 7.4.
2. Platinum wire loop.

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3. Microscope with oil immersion lens and immersion oil.
4. Bunsen burner.
5. Two glass microscope slides.
6. Distilled water.
7. Gram stain equipment.

*Technique.*—The test, to be done at a temperature not above 50° C., consists of the following steps:

1. To four drops of fresh 10 per cent sodium desoxycholate on the first glass slide (*I*) add two medium-sized loopful of the material containing the organism to be tested (washed sputum, broth suspension of the organism, or colonies from agar plate). Allow the slide to stand for ten minutes and shake gently occasionally.

2. On one-half of the second glass slide (*II*) make a direct smear from the original material as a control (*C*).

3. After ten minutes, to one drop of distilled water on the other half of the second glass slide (*II*), add one loopful of material from the mixture of the first glass slide as the test (*T*).

4. Dry this second glass slide (preferably on a warm ring stand), Gram stain, and compare the control smear with the test smear under oil immersion. The absence on the test smear of gram-positive encapsulated organisms, such as are present on the control smear, indicates a positive test. The presence of such organisms on both test and control smears indicates a negative test; in other words, the organisms tested are not pneumococci.

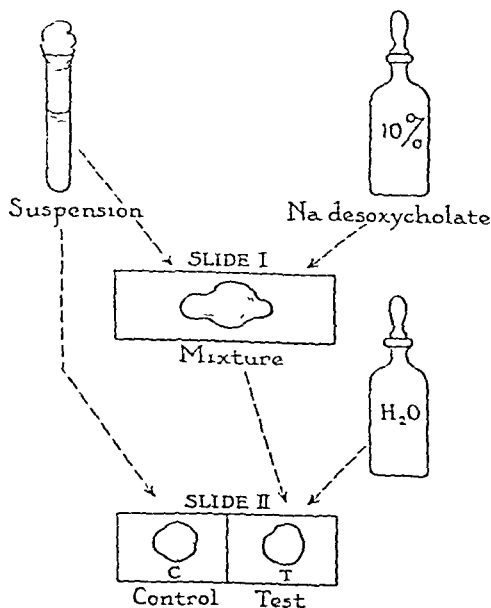


Fig. 1.—Diagram of procedure.

#### RESULTS

This method was used in testing six different types of pneumococci previously identified by serologic typing and one strain of gamma nonhemolytic streptococcus. In Fig. 2 the results obtained by our method are compared with those obtained by Liefson's method in fifteen cases. In one of these cases there

was only partial solution of the pneumococci with our method. Using Liefson's technique we found complete solubility in one case and no appreciable solubility in the others.

As a means of checking our technique, we compared its results with those obtained by the standard macroscopic method described by Kolmer and Boerner<sup>6</sup> in a series of twenty-five different types of pneumococci in pure culture. Complete solubility occurred by both methods in all the types tested.

Repeated success with our method during the year in a series of over 200 cases, and covering more than 500 separate tests, constitutes further indication of its accuracy. In no instance in seventy-seven cases of known streptococcal infections tested have we ever dissolved the alpha, beta, or gamma streptococci by this method.

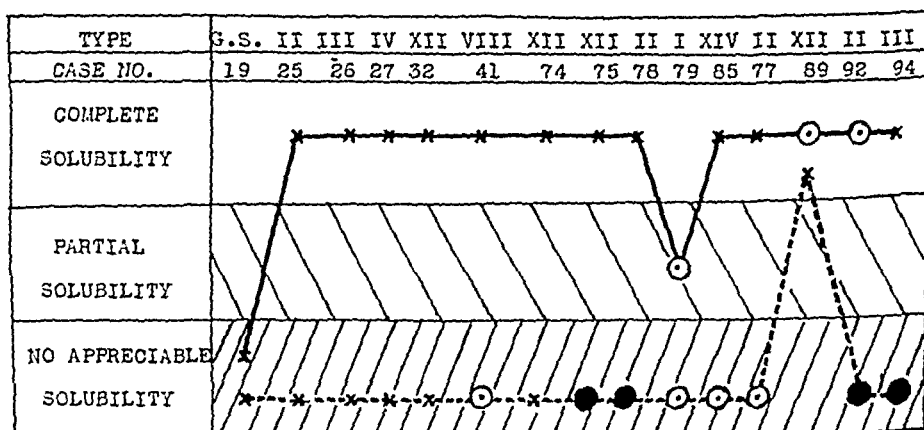


Fig. 2.— - - - - Liefson's method. — Slide method. ○, "Quellung shadows" observed with the addition of type-specific serum. ●, "Quellung shadows" and also ordinary "Quellung phenomenon" observed with the addition of type-specific serum. G. S., Gamma streptococcus; roman numerals refer to pneumococcus types.

In the course of this work the observation was made that if the test (*T*) was not allowed to dry, but instead type-specific serum was added directly to the moist preparation, the presence of enlarged capsules or "Quellung shadows" were noted eight times in Liefson's test and three times in our test in the representative series (Fig. 2). The bodies of the organisms were apparently dissolved by the bile salt and the capsules remained intact. The intact capsules were invisible until type-specific serum was used and the test was examined under oil immersion with a dull light; the capsules appeared translucent and took no stain, whereas the body, or normally staining portion, was absent.

#### COMMENT

In a study of twenty-seven pneumococcal infections, 77 streptococcal infections, and 11 miscellaneous infections, the slide test described in this report served as an accurate method of identifying pneumococci in spinal fluid, washed sputum, and in either pure or mixed cultures grown on blood or proteose agar media. In the latter instance, when the contaminating organisms were alpha

hemolytic streptococci, we found that the addition of the six groups of Neufeld typing sera served to identify the pneumococci by "Quellung shadows" and the alpha hemolytic streptococci as undissolved intact organisms.

The presence of the "Quellung shadows" suggests that proteolysis occurs in the presence of excess sodium desoxycholate. Goebel and Avery<sup>7</sup> have shown that autolysis is accompanied by proteolysis and lipolysis, whereas sodium desoxycholate, in excess, inhibits the action of pneumococcal protease, but not lipase. These authors, however, found that pneumococcus is dissolved in the presence of an excess of the bile salt and concluded that bile solubility is probably not identical with autolysis. We also found that the body of the organism is dissolved even though the protease is apparently inhibited in the presence of excess sodium desoxycholate. Further, we found an apparent resistance at times of the carbohydrate or capsular portion of the pneumococcus to the action of excess sodium desoxycholate as was clearly shown by the "Quellung shadow" phenomenon.

#### SUMMARY

1. A simple modification of the bile solubility test is described.
2. The presence of the "Quellung shadows" in bile-tested material from mixed cultures is suggested as a further aid in the differentiation between pneumococci and alpha hemolytic streptococci.
3. The significance of the "Quellung shadows" is discussed.

#### REFERENCES

1. Neufeld, F.: Ueber eine spezifische bakteriolytische Wirkung der Galle, *Ztschr. f. Hyg. u. Infektionskrankh.* 34: 454, 1900.
2. Nicolle, M., and Adil-Bey: Action de la bile sur le Pneumocoque, et diverses autres bacteries. *Ann. de l'Inst Pasteur* 21 (1): 20, 1907.
3. Dochez, A. R., and Gillespie, L. J.: A Biologic Classification of Pneumococci by Means of Immunity Reactions, *J. A. M. A.* 61: (10): 727, 1913.
4. White, B., Robinson, E. S., and Barnes, L. A.: *The Biology of Pneumococcus*, Commonwealth Fund, London, 1938, Oxford University Press, pp. 55-62.
5. Liefson, E.: Use of Sodium Desoxycholate for Identification of Pneumococci, *J. A. M. A.* 104: 213, 1935.
6. Kolmer, J. A., and Boerner, F.: *Approved Laboratory Technic*, ed. 4, New York, 1945, D. Appleton-Century Company, Inc., pp. 392-393.
7. Goebel, W. F., and Avery, O. T.: A Study of Pneumococcus Autolysis, *J. Exper. Med.* 49 (2): 267, 1929.



# A SIMPLE APPARATUS FOR CULTURING NEISSERIA GONORRHEA UNDER PARTIAL CARBON DIOXIDE TENSION

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**A**N EVER-INCREASING demand for the isolation and identification of *Neisseria gonorrhoea* by culture methods, not only as an aid to diagnosis, but also as a criterion for cure of both male and female patients following chemotherapy, makes it desirable that a simple apparatus for culturing gonococci be made available.\*

The following simple apparatus has been devised to meet this demand and has proved to be readily applicable for routine gonococcus culture methods.

The apparatus consists of a standard laboratory desiccator (Scheibler type, 150 mm.), having an approximate capacity of 2,500 c.c. and a plexiglass Petri dish holder, so constructed to accommodate either a medicine dosage glass or a porcelain crucible as a reaction vessel for the production of carbon dioxide by the action of dilute HCl with sodium bicarbonate.

The Petri dish holder (Fig. 1) is constructed from the ordinary plastic, plexiglass ( $\frac{1}{4}$  inch thickness), and the component parts of the holder (top, base, and three supports) are cemented together with an adhesive made by dissolving plastic shavings in acetone. Increased rigidity of the culture plate holder is accomplished by dovetailing the supports at the top and base. Since the culture plate holder fits snugly in the desiccator, a series of holes (0.4 cm.) are drilled in the top and base plates to insure adequate circulation of  $\text{CO}_2$  throughout the desiccator. Sufficient  $\text{CO}_2$  tension is produced by the addition of 3 or 4 c.c. of 10 per cent HCl to approximately 1 Gm. of sodium bicarbonate.

The following procedure is employed in preparing the apparatus for incubation. After the culture plates have been inoculated, they are placed in the culture plate holder, which is then introduced into the desiccator. The reaction vessel containing the sodium bicarbonate (Fig. 2, A) is placed in the round opening on the top plate of the culture dish holder. The lid of the desiccator is tightly sealed in place, leaving the stopcock open. Dilute HCl is then delivered with a 5 c.c. diluting pipette through the open stopcock into the reacting vessel. The stopcock is immediately closed and the entire apparatus is placed in a bacteriologic incubator for the desired length of incubation at  $37^\circ$  centigrade.

The advantages of an all-glass culture apparatus, plus the noncorrosive plexiglass culture plate holder, are obvious when one remembers the disagreeable features of using a copper Petri dish holder utilizing a burning candle for the production of carbon dioxide. The previously described apparatus can easily be cleaned and sterilized after each incubation period. If additional moisture is

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\*The apparatus was devised and used by the author while serving as technical sergeant, Medical Department, in the Armed Forces of the United States.

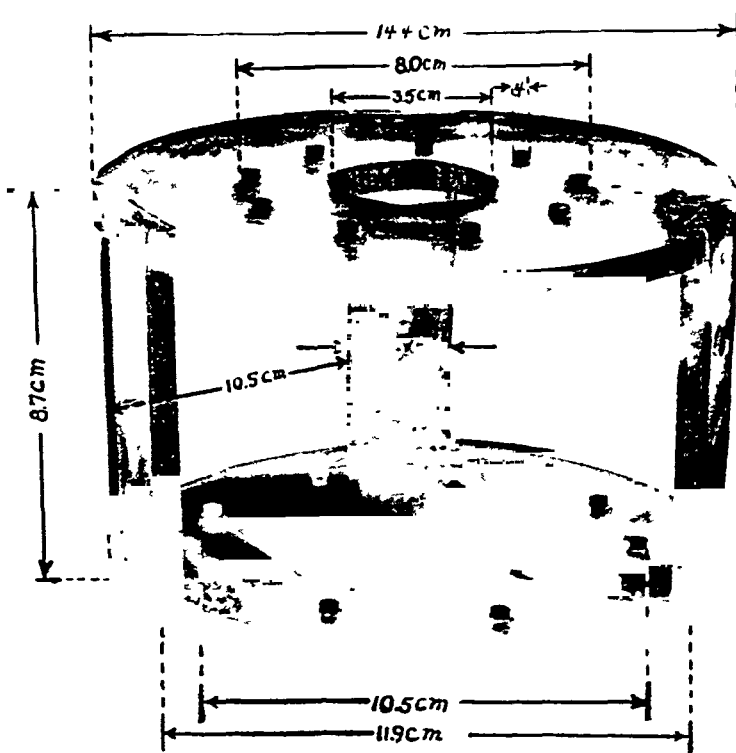
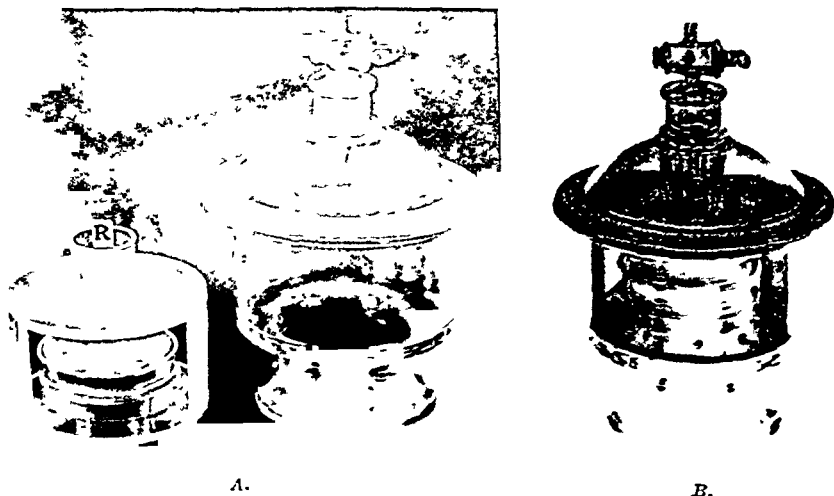


Fig. 1.—Plexiglass Petri dish holder, with a 3.5 cm. hole in the top plate to accommodate either a medicine dosage glass or porcelain crucible as a reaction vessel for the production of carbon dioxide.



A.

B.

Fig. 2.—A, View of the culture apparatus, showing the reaction chamber (R) and inoculated culture plates ready for introduction into the desiccator. B, Assembled gonococcus culture apparatus ready for incubation.

necessary to safeguard against dehydration of the culture media during incubation, a very small amount of water can be placed on the floor of the desiccator before the culture plate holder is introduced into the desiccator.

The apparatus has been used by the author for more than one year and found satisfactory for routine gonococci cultures, using either chocolate-agar (Difco) or Peizer media.

#### SUMMARY

A simple apparatus for culturing *Neisseria gonorrhoea* under partial carbon dioxide tension is described. It has been found to be adequate for the culture of gonococci as an aid to diagnosis.

# A TEST FLUID FOR CONTROL OF THE GOLD REACTION

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CORRECT results in the colloidal gold test depend primarily upon two physicochemical factors<sup>1</sup>: the first is the pH of the milieu,<sup>2</sup> which is optimal at 7.4, and the second is the sensitivity (dispersity) of the gold sol, which should be controlled by use of a photoelectric colorimeter.<sup>3</sup> A test fluid is required as an additional safeguard to demonstrate that optimal conditions have been secured.

Such a test fluid may be prepared by pooling approximately equal amounts of twenty or more normal sera. Specimens that are chylous or otherwise abnormal in appearance, or that react in the complement-fixation test for syphilis, are excluded. Additional criteria for the "normality" of the sera are not used. Exactly 5 ml. of the pooled sera are diluted with phosphate buffer, pH 7.4,<sup>4</sup> in a 250 ml. volumetric flask. Fifty milliliters of this solution are diluted with 50 ml. of glycerol, the final dilution being 1:100. The fluid is kept in the refrigerator and is freshly prepared each month. One-tenth milliliter is tested in duplicate every working day in the same manner as a specimen of cerebrospinal fluid.

The test fluid when satisfactory yields a uniformly standard curve characterized by three factors: (1) The degree of coagulation, determined by the gold color standard, is 6 in the first or 1:15 dilution; (2) the maximum reaction, 15, occurs in dilution 1:114; and (3) the sum of the ten numerical color values is between 95 and 100. The exact reproducibility of the test fluid was demonstrated by testing ten different serum pools at one time with the same reagents. The results were identical with reference to the three factors mentioned. The negligible deviations in other dilutions, particularly in the postzone, are not due to differences of the test fluid but to inevitable slight inaccuracies of pipetting. To obtain such results, the dilution of the serum pool (1:100) must be exact. Experience has shown that on rare occasions an abnormal serum that has been inadvertently included may spoil a pool.

The standard curve is the final criterion of the suitability of the test fluid and other reagents and particularly of the gold color standard. In fact, it provides a rigorous control of the test as a whole. Deviations from the standard curve indicate that a reagent is unsatisfactory. The source of the deviation is determined by replacing one reagent at a time, which can be done easily if a set of reagents known to be satisfactory is available. For example, if a new buffer is substituted and the test fluid does not give the standard curve, the new buffer solution is evidently at fault.

Use of the test fluid is illustrated in Table I. In the first four items, in which the colloidal gold solutions are of satisfactory sensitivity as indicated by the turbidimetric value, the pH of the milieu is 7.4 by potentiometric determination and the buffer is of satisfactory pH and molar concentration. The

From the Division of Laboratories and Research, New York State Department of Health. Received for publication, March 13, 1947.

TABLE I. COLLOIDAL GOLD REACTIONS WITH TEST FLUID PREPARED FROM POOL OF NORMAL SERA

SERIAL NUM- BER	COLLOIDAL GOLD SOLU- TIONS* (DATE OF PREPARA- TION)	TURBID- ITY	TEST FLUID PREPARED	PH OF MILIED†	MOLAR- ITY	COLLOIDAL GOLD REACTIONS NEW SERIAL DILUTIONS										SUM TOTAL
						1:15	23	31	51	76	111	171	256	384	576	
1	6/25/46	135	6/13/46	7.4	M/21.5	6	7.5	8.5	12	13	15 <sup>1</sup>	14	9	7	6.5	99 Standard curve; every reagent correct
2	6/ 6/46	122	6/13/46	7.4		6	7	9	11	13	15 <sup>1</sup>	14	8.5	6	5.5	93 Standard curve; every reagent correct
3	2/28/46	128	2/18/46	7.4		6	7.5	9.5	13	14	15 <sup>1</sup>	14	9	6.5	5	100 Standard curve; every reagent correct
4	2/28/46	128	3/ 4/46	7.4		6	8	9.5	13	14	15 <sup>1</sup>	14	10	7	3	100 Standard curve; every reagent correct
5	6/25/46	135	6/13/46	5.2	M/20	9	13	15	16	18	18	18	18	16	16	157 Inadequate pH of milieu
6	6/25/46	135	6/13/46	6.0	M/20	8	9	12	14	14	9.5	9	6	5	4	91 Inadequate pH of milieu
7	6/25/46	135	6/13/46	6.4	M/20	7	8	10	13	14	14	9	7	5.5	4	92 Inadequate pH of milieu
8	6/25/46	135	6/13/46	7.4	M/ 5.4	8.5	15	18	18	18	18	18	18	18	18	168 Inadequate electro- lyte concentra- tion
9	3/27/46	73	3/ 4/46	7.4	M/21.5	4.5	6.5	9.5	13	14	15 <sup>1</sup>	14	9.5	7	4.5	98 Inadequate sensi- tivity of gold sol
10	12/13/46	70	10/22/46	7.4	M/21.5	3.5	6	9	10	13	11	7	6	4.5	3	73 Inadequate sensi- tivity of gold sol

\*Citrate gold 1:10,000<sup>3</sup> was used.†The routine phosphate buffer pH 7.4 was used except in the case of Nos. 1, 6, and 7 in which phthalate buffer pH 5.2, pH 6.0, and pH 6.4, respectively, were used.  
if the  $\Delta$  indicates the location of the maximum reaction in prezone curves.

findings obtained are almost identical, although the gold sols were prepared on different days and different test fluids were used. When the pH of the milieu is more acid than 7.4, the results vary markedly from those obtained when the pH is maintained at 7.4, as in items 5, 6, and 7. When the molar concentration of the buffer is incorrect (item 8), the results again show clearly that a mistake has been made. The pH and the molar concentration are controlled simultaneously by the test fluid, which is not possible by potentiometric determination.

In items 9 and 10, citrate gold sols 1:10,000 prepared with impure distilled water were used. The turbidity and sensitivity of these sols were abnormally low. Below a certain limit, abnormality is clearly demonstrable by the test fluid. In both Nos. 9 and 10, the degree of coagulation in the prezone is abnormal; in No. 10, even the location of the maximum is shifted and the sum total is markedly decreased.

Two controls for particle size and reproducibility of the test as a whole (turbidimetric determinations and use of a test fluid) are recommended. A gold sol having an abnormal particle size can be detected by turbidimetry. Such findings indicate the use of impure distilled water. The test fluid, however, shows in detail the type and degree of deviation from the standard reaction.

#### OTHER TEST FLUIDS

Before pools of normal sera were found to provide a satisfactory test fluid, the value of other types of fluid was studied as follows:

1. Four cerebrospinal fluids of different protein patterns (types A, B, C, and D), preserved by adding equal amounts of glycerol, were used. If the pH and the sensitivity were correctly adjusted, reproducible results were obtained. It was difficult, however, to obtain four suitable fluids for the purpose, and when a satisfactory specimen was obtained, only a small amount was available.

2. Use of cerebrospinal fluids from patients with paresis<sup>6</sup> is unsatisfactory. Only test fluids yielding a prezone curve, such as types B or C,\* can demonstrate that a false D (paretic) curve<sup>2</sup> has been obtained if the pH is too acid.

3. Purified globulin fractions yield either plateau (D) or prezone (B or C) curves. Even if the pH of the milieu is maintained at 7.4, test fluids giving a plateau (paretic or type D) curve are inadequate. A solution of 20 mg. per 100 ml. of gamma globulin was found to yield the same plateau or first-zone curve as a specimen of cerebrospinal fluid from a paretic. A plateau of complete coagulation was obtained with both, up to a final dilution of 1:320. A solution of 10 mg. per 100 ml. of globulin gave a plateau of complete coagulation in a 1:160 dilution. A solution of 15 mg. per 100 ml., however, could not be differentiated by end-point titration from a solution of either 10 or 20 mg. per 100 ml. Hence, test fluids that yield a plateau curve, besides being inadequate as a control of the pH, provide only a rough control in the quantitative adjustment or sensitivity.

\*Classification of curves obtained in the colloidal gold test: A. Obtained with normal cerebrospinal fluid; B, formerly designated as second zone or syphilitic; C, formerly designated as hematogenous, third zone, or meningitic; D, formerly designated as paretic or first-zone curve.

4. Edestin,<sup>7</sup> widely used as a test fluid, yields a first-zone curve and in some respects is even inferior to gamma globulin.

5. Pseudoglobulin obtained from normal horse serum by repeated fractionation, between 33 and 45 per cent saturation with ammonium sulfate, produces a prezone curve. It provides a highly satisfactory control on quantitative and qualitative reproducibility of the gold reaction. Its preparation is somewhat complicated, however, and the reproducibility of the test fluid itself is unsatisfactory.

#### SUMMARY

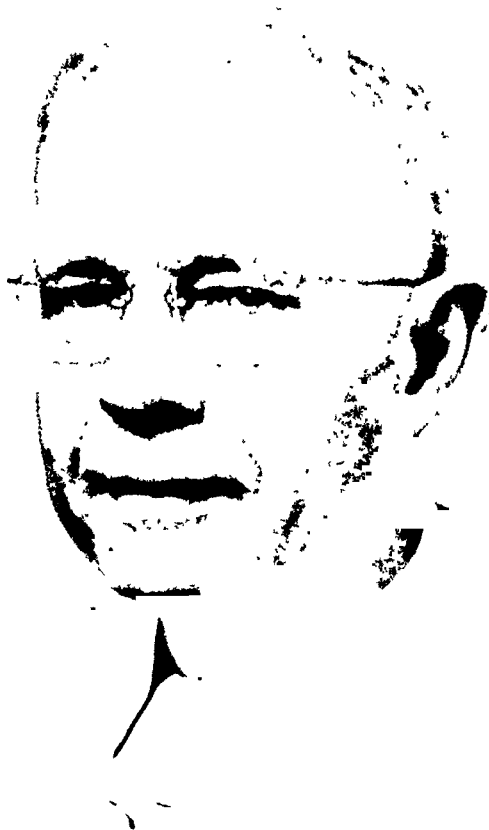
A test fluid prepared from pools of normal sera for use as a control in the colloidal gold test is described. The fluid is simple to prepare and reproducible. The procedure recommended permits identical results to be obtained in different laboratories and is a step forward in standardization of the gold reaction.

#### REFERENCES

1. Lange, C.: Theory of the Colloidal Gold Reaction, *J. LAB. & CLIN. MED.* 30: 1006-1012, 1945.
2. Lange, C., and Harris, A. H.: The Significance of the pH in the Gold Reaction, *J. LAB. & CLIN. MED.* 29: 970-975, 1944.
3. Lange, C., and Harris, A. H.: A Citrate Gold of Optimal and Reproducible Sensitivity for Use in the Colloidal Gold Reaction, *Am. J. Pub. Health.* 34: 1087-1092, 1944.
4. Lange, C.: Methods for the Examination of Spinal Fluid, *Am. J. Syph., Gonorr. & Ven. Dis.* 23: 638-663, 1939.
5. Lange, C.: Die Ausflockung kolloidalen Goldes durch Cerebrospinal flüssigkeit beiluetischen Affektionen des Zentralnervensystems, *Ztschr. f. Chemotherapie.* 1: 44-78, 1912.
6. Adams, D. K., and Scott, W. H.: The Colloidal Gold Reaction of Cerebrospinal Fluid; Standardising and Application in Early Nervous Disease, *J. Path. & Bact. (Proc. Soc.)* 25: 142-143, 1922.
7. Kreidler, W. A., and Small, J. C.: A Method of Standardizing Colloidal Gold Sols by Utilizing a Standard Solution of Globulin, *J. LAB. & CLIN. MED.* 17: 269-271, 1931.







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## IN HONOR OF DR. HAL DOWNEY

**M**Y FRIEND, Professor Hal Downey, retired last June from active duty on the teaching faculty of the University of Minnesota. This event prompted former students and associates to dedicate this issue of the JOURNAL OF LABORATORY AND CLINICAL MEDICINE to him as a mark of esteem and gratitude. They asked me to join them in this token of respect.

I am one of the few survivors of the old school of nonclinical hematology and had the privilege of working with Dr. Downey when he visited Europe in 1911, with the intention of extending his earlier studies on the blood-forming organs of the lower vertebrates (*Polyodon* in particular) to mammals and man. He went first to Pappenheim in Berlin. But Pappenheim's more philosophical mind and mode of viewing morphological problems were not entirely to Downey's taste. So he left Berlin and went to Strassburg to work in my laboratory. The equipment there was very poor and no technical assistance was available. We had only the simplest microscopical techniques with which to tackle the problem of the origin of the lymphocytes—which was our goal.

When Downey left me, I gave up hematology and never returned to it. Downey, however, remained faithful to the subject. Acknowledging that the clue to the lymphocyte problem, the relation between lymphoblasts and myeloblasts, is only approachable through experiment—that is, changing the normal environment of the cells—he decided to study the blood cells under their pathological conditions.

I do not feel qualified to praise Dr. Downey's achievement in the field of clinical hematology and must leave this to more competent persons. But I am very proud of having served Dr. Downey as a guide at the beginning of a career in which he has become one of the foremost hematologists of our age.

—*Franz Weidenreich.*

# AN EVALUATION OF ALTERATIONS PRODUCED IN LYMPHOID TISSUE BY PITUITARY-ADRENAL CORTICAL SECRETION

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THE involvement of lymphoid tissue and of lymphocytes in numerous normal and pathologic processes is well recognized by pathologists and experimental investigators. However, the relation of the experimental and pathologic findings to the actual function of the lymphocyte has remained obscure. The normal physiologic control and function of this ubiquitous tissue has been largely speculative. Perla and Marmorston,<sup>1</sup> in discussing lymphoid tissue, ask the following questions: "Why does the mammal possess so large and diffuse an organ (lymphoid tissue)? What is the purpose of this mass of lymphoid tissue? Why are such large numbers of lymphocytes poured into the circulation daily, without causing a fluctuation in the lymphocytic count of the blood? Where do the lymphocytes go? What is the function of the lymphocyte?"

Drinker and Yoffey<sup>2</sup> have stated that the only definitely known function of lymphoid tissue is the production of lymphocytes. Actually, the origin of the lymphocyte is still a matter of controversy among some histologists. The multipotentiality of the lymphocyte has been demonstrated.<sup>3</sup> Lymphocytes may be sources of immature cells, which transform to specialized cellular forms when the necessity arises, or a reservoir of potential macrophages. However, these roles are potentialities of the lymphocyte; the normal functions of the cell morphologically identified as a lymphocyte have not been elucidated.

The constancy in the size of the lymphoid tissue and in the numbers of circulating lymphocytes suggest the possibility that the rate of growth of lymphocytes and their entry into the circulation may be under endocrine control. It is not unlikely that these processes may be intimately related also to factors concerned with the peripheral disposition of lymphocytes. Addison noted an increase in lymphoid tissue in his original description of the disease which bears his name (quoted by Grollman<sup>5</sup>). Conversely, numerous investigators have found that the secretions of the adrenal cortex have a regulatory effect on the size of the thymus.<sup>6-12</sup> This paper includes some of the data obtained in studies initiated to investigate the hypothesis that pituitary adrenotrophic hormone (adrenotrophin), by its action on the adrenal cortex, influences the size, structure, and function of lymphoid tissue.

## EXPERIMENTAL OBSERVATIONS

*Pituitary-Adrenal Cortical Control of Lymphoid Tissue Weight.*—The daily subcutaneous injection of 1 mg. of adrenotrophin<sup>13</sup> in 0.5 c.c. aqueous solution

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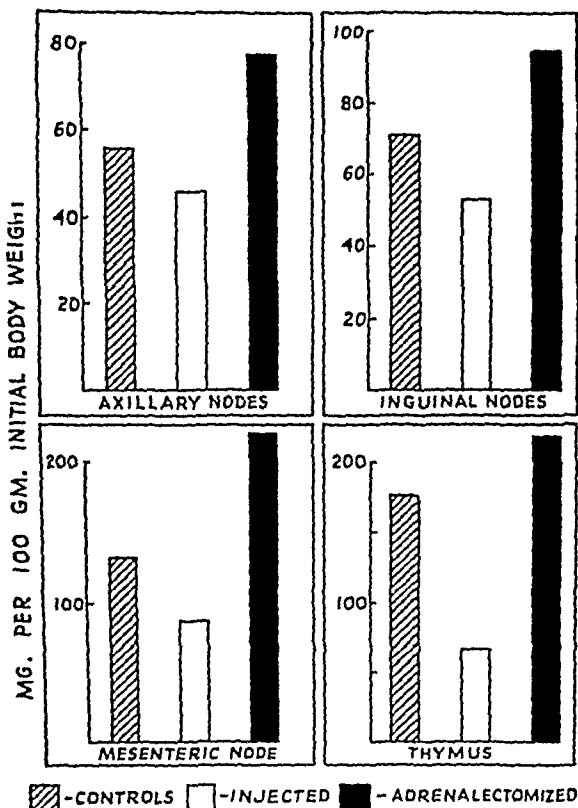
into normal CBA mice, 60 to 80 days old, resulted in a significant decrease in the weight of lymphoid tissue. This decrease was observed as early as fifteen hours following injection and persisted throughout the fifteen-day period during which the mice were treated<sup>14</sup> (Figs. 1 and 2). The thymus remained smaller than other lymphoid structures during the period of study. Although the spleen actually increased in weight during the fifteen days of hormone administration, this increase was due to the accumulation of large numbers of degenerating lymphocytes and to an extensive edema. The Malpighian corpuscles were markedly decreased in size. The histologic changes in these tissues will be discussed later. Adrenotrophin has now been reported to produce similar weight changes in lymphoid organs of the rat.<sup>15</sup>

Adrenalectomized mice maintained for eight days by daily, subcutaneous injections of 0.025 mg. desoxycorticosterone acetate in oil (Schering), showed marked hyperplasia of all the lymphoid organs studied (Figs. 1 and 2). The alterations in lymphoid tissue following adrenalectomy were not influenced by desoxycorticosterone acetate since the maintenance dose of this steroid produced no effect on the weight or histology of lymphoid tissue in normal or adrenalectomized mice.<sup>16, 17</sup>

*Pituitary Control of Adrenal Size and Cholesterol Content.*—Repeated daily injections of adrenotrophic hormone resulted in the expected increase in weight of the adrenals (Fig. 2). The cholesterol content of the adrenals of hormone-treated mice varied during the fifteen-day period, depending on the particular day the glands were obtained. Whereas a single injection of hormone produces an acute decrease in adrenal cholesterol within a few hours after administration<sup>18</sup> (Fig. 3), repeated daily injections increase the adrenal cholesterol to values above normal.<sup>19</sup>

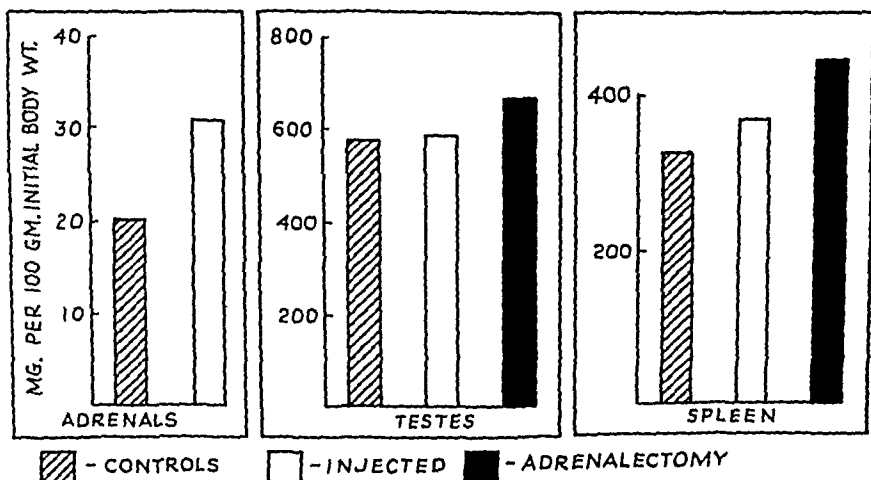
*Pituitary-Adrenal Cortical Control of the Numbers of Circulating Lymphocytes.*—In the fifteen-day experiments previously described animals were sacrificed in groups at intervals of three days. All of these mice were killed approximately twenty-four hours after the last injection of adrenotrophic hormone. In addition to weighing the tissues, red and white blood cell counts and differential counts were made. Total hemoglobin and whole blood specific gravity were also determined. The data are summarized in Table I. The number of circulating lymphocytes in the adrenotrophic hormone-treated mice was significantly lower throughout the fifteen-day period.<sup>20</sup>

Similar studies have been conducted on adrenalectomized mice maintained on daily injections of 0.025 mg. desoxycorticosterone acetate over an eight-day period and on adrenalectomized rats given 0.7 per cent sodium chloride and 0.3 per cent sodium bicarbonate as drinking fluid. These data are presented in Tables I and II. In adrenalectomized mice and rats the number of lymphocytes increased significantly.<sup>20</sup> In the rats there was a significant lymphocytosis at six days following adrenalectomy, whereas mice had a lymphocytosis twenty-four hours after operation. Furthermore, the specific gravity of the whole blood of adrenalectomized mice maintained with desoxycorticosterone did not indicate a hemoconcentration sufficient to account for the increased number of lymphocytes (Table I). The number of circulating lymphocytes in adrenalectomized



EFFECT OF ADRENOTROPIC HORMONE INJECTION AND ADRENALECTOMY ON TISSUE WEIGHTS OF THE MOUSE

Fig. 1.—The columns represent weights of paired lymph nodes, adrenals, and testes of mice, 60 to 80 days old, of the CBA strain (Strong). The control group represents thirty mice, and there were twenty-five mice in each of the experimental series. The adrenotropic hormone-injected mice were treated daily with 1.0 mg. of hormone for fifteen days; the adrenalectomized mice were sacrificed one week after adrenalectomy.



EFFECT OF ADRENOTROPIC HORMONE INJECTION AND OF ADRENALECTOMY ON TISSUE WEIGHTS OF THE MOUSE

Fig. 2.—Same as Fig. 1.

TABLE I. EFFECT OF CONTINUED INJECTION OF ADRENOTROPHIC HORMONE AND OF ADRENALCTOMY ON CERTAIN BLOOD CONSTITUENTS OF THE MOUSE\*

	ADRENOTROPHIC HORMONE-INJECTED ANIMALS		ADRENALCTOMY ANIMALS		ADRENALCTOMY ANIMALS + DCA	
	CONTROL—UNINJECTED ANIMALS	1 MG. DAILY 15 DAYS	ADRENALCTOMY ANIMALS	ADRENALCTOMY ANIMALS	ADRENALCTOMY ANIMALS + DCA	P VALUES†
Number of animals	99	30	14	32		
Hemoglobin (Gm. %)	15.3 ± 0.624	16.4 ± 0.25	17.5 ± 0.27	15.9 ± 0.18		0.06
R.B.C. (mil./c.mm.)	9.05 ± 0.50†	10.15 ± 0.19	9.73 ± 0.47	9.54 ± 0.25		0.10
W.B.C.	12,814 ± 435	11,749 ± 737	17,025 ± 1,682	15,761 ± 819		< 0.01
Lymphocytes	9,584 ± 371	6,932 ± 510	14,549 ± 1,514	11,674 ± 684		< 0.01
Polymorphonuclear leucocytes	3,257 ± 158	4,694 ± 358	2,476 ± 287	3,087 ± 397		0.6
Specific gravity whole blood	1.060 ± 0.0006§	1.063 ± 0.0058	1.068 ± 0.0013	1.064 ± 0.0002		< 0.01

\*Values in this table are means and standard errors.

†P values as compared to controls.

‡Thirty-nine animals.

§Nineteen animals.

||Ten animals.

TABLE II. EFFECT OF ADRENALCTOMY ON CERTAIN BLOOD CONSTITUENTS OF THE RAT\*

	CONTROL—UNINJECTED ANIMALS		TWO DAYS AFTER ADRENALCTOMY		FOUR DAYS AFTER ADRENALCTOMY		SIX DAYS AFTER ADRENALCTOMY	
	CONTROL—UNINJECTED ANIMALS	ADRENALCTOMY ANIMALS	ADRENALCTOMY ANIMALS	ADRENALCTOMY ANIMALS	ADRENALCTOMY ANIMALS	ADRENALCTOMY ANIMALS	ADRENALCTOMY ANIMALS	ADRENALCTOMY ANIMALS
Number of animals	42	13	9	9	9	9	9	9
Hemoglobin (Gm. %)	14.8 ± 0.14†	14.0 ± 0.35	14.0 ± 0.35	13.4 ± 0.37	12.8 ± 0.38	12.8 ± 0.38	12.8 ± 0.38	12.8 ± 0.38
R.B.C. (mil./c.mm.)	8.88 ± 0.23†	9.38 ± 0.31	9.38 ± 0.31	8.02 ± 0.18	7.93 ± 7.73	7.93 ± 7.73	7.93 ± 7.73	7.93 ± 7.73
W.B.C.	17,826 ± 809	17,194 ± 1,748	17,194 ± 1,748	19,011 ± 2,016	26,082 ± 1,825	26,082 ± 1,825	26,082 ± 1,825	26,082 ± 1,825
Lymphocytes	11,700 ± 724	13,946 ± 1,182	13,946 ± 1,182	15,739 ± 1,597	20,011 ± 1,201	20,011 ± 1,201	20,011 ± 1,201	20,011 ± 1,201
Polymorphonuclear leucocytes	2,932 ± 734	4,163 ± 274	4,163 ± 274	3,272 ± 839	6,072 ± 921	6,072 ± 921	6,072 ± 921	6,072 ± 921
Specific gravity whole blood	1.050 ± .00004§	1.058 ± .001	1.058 ± .001	1.057 ± .0009	0.056 ± .0002	0.056 ± .0002	0.056 ± .0002	0.056 ± .0002

\*Values in this table are means and standard errors.

†P values as compared to controls.

‡Twenty-seven animals.

§Eight animals.

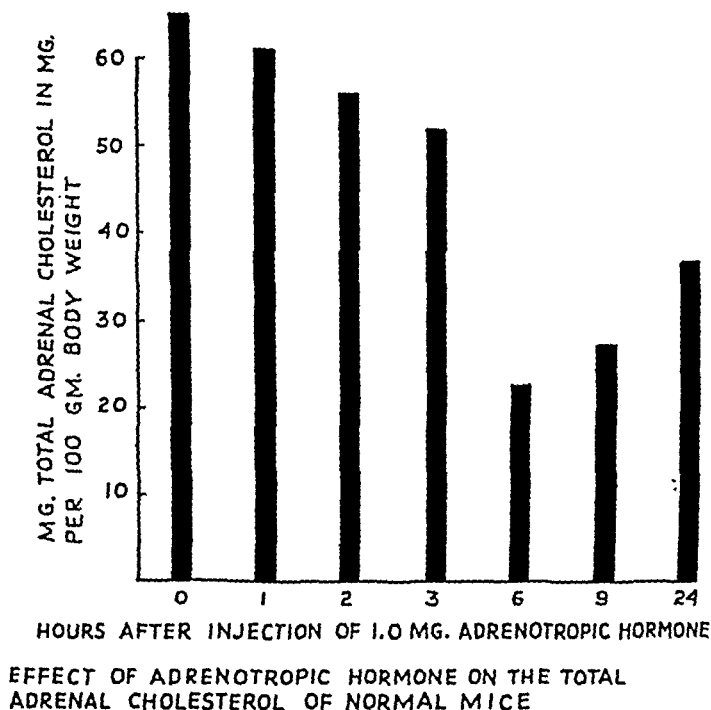


Fig. 3.

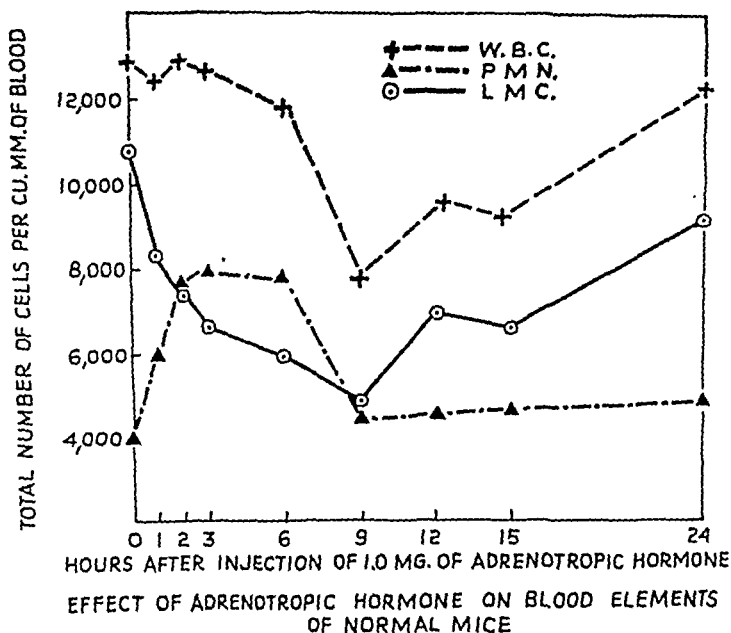


Fig. 4.—Alterations produced in total leucocyte, lymphocyte, and polymorphonuclear cells in blood of normal mice receiving at zero time a single subcutaneous injection of 1.0 mg. of pituitary adrenotropic hormone. The averages at each successive time interval are for groups of 99, 3, 3, 7, 7, 7, 6, 7, and 7 animals.

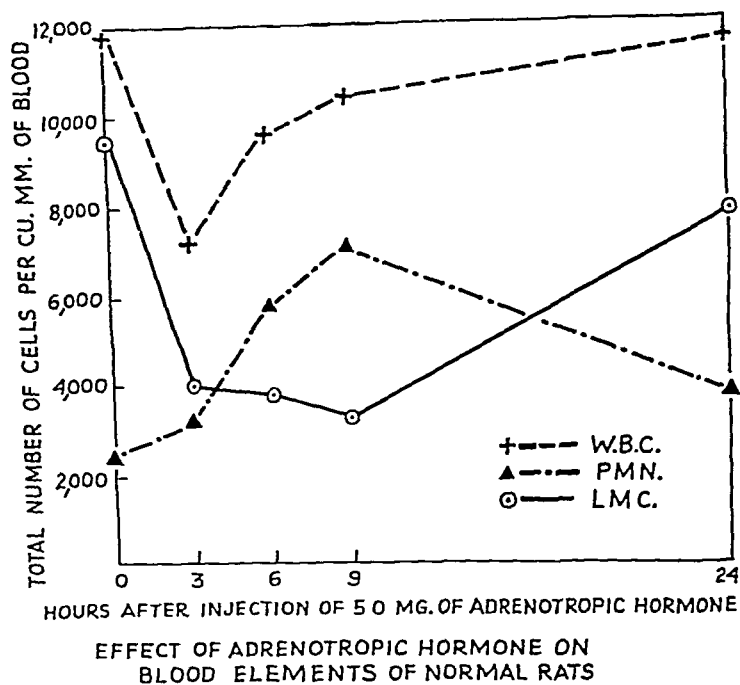


Fig. 5—Alterations produced in total leucocyte, lymphocyte, and polymorphonuclear cells in blood counts of normal rats receiving at zero time 5.0 mg. of adrenotropic hormone subcutaneously. The averages at each successive time interval are for groups of 42, 12, 12, 8, and 12 animals

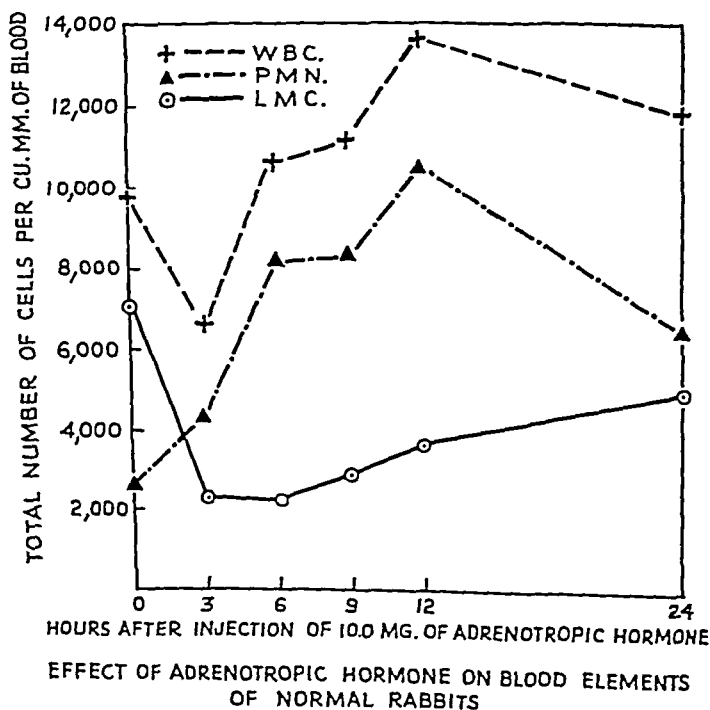


Fig. 6—Alterations produced in total leucocyte, lymphocyte, and polymorphonuclear cells in blood counts of normal rabbits receiving at zero time 10.0 mg. of adrenotropic hormone subcutaneously. The averages at each successive time interval are for groups of 18, 4, 4, 4, 2, and 3 animals.



rats provides further evidence that the increase in lymphocyte counts in the operated mice is not due to hemoconcentration, since the adrenalectomized rats had an approximately normal blood volume and yet showed a lymphocytosis (Table II).

*Significance of Lymphocyte Counts as an Index of Adrenal Cortical Activity.*—The slight, but significant, lymphopenia in mice twenty-four hours after the previous injection of adrenotrophin suggested that the numbers of circulating lymphocytes might be an index of the level of adrenal cortical activity. On the basis of the adrenal cholesterol analyses shown in Fig. 3, maximum adrenal cortical activity occurred at six hours following a single injection of adrenotrophic hormone. Therefore, the blood of mice, rats, rabbits, and dogs was studied at intervals shortly following subcutaneous injection of this hormone.

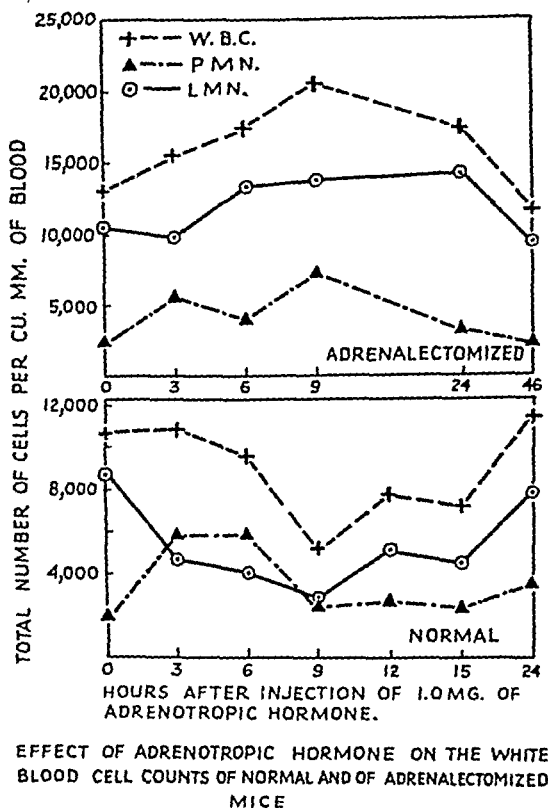


Fig. 7.—Alterations produced in the leucocytes of adrenalectomized mice receiving at zero time 1.0 mg. of adrenotropic hormone subcutaneously. The lower chart for normal animals is reproduced for comparison. The averages represent sixty control animals at zero time and five at each successive interval.

A single subcutaneous injection of 1.0 mg. of adrenotrophin in mice produced a precipitous decline in the numbers of circulating lymphocytes which was evident within one hour and reached its lowest point within nine hours<sup>18</sup> (Fig. 4). Following this time, the numbers of blood lymphocytes rapidly increased and at twenty-four hours after hormone administration had returned

almost to normal. The lymphopenic response also was observed in rats injected with 5 mg. of adrenotrophic hormone (Fig. 5) and in dogs given 10 mg. of the hormone. In rabbits receiving 10 mg. of adrenotrophin the numbers of lymphocytes again decreased very rapidly, reaching their lowest value within three hours, and did not return to normal levels as rapidly as in the other species (Fig. 6).

The lymphopenic response produced by adrenotrophic hormone is dependent upon the mediation of the adrenal cortex. The hormone has no effect on blood lymphocytes in adrenalectomized animals (Fig. 7). Further, the specific hormonal action of adrenotrophin is seen from the fact that two other pure proteins, prolactin<sup>21</sup> and serum gamma globulin (human), had no effect on blood lymphocyte levels.

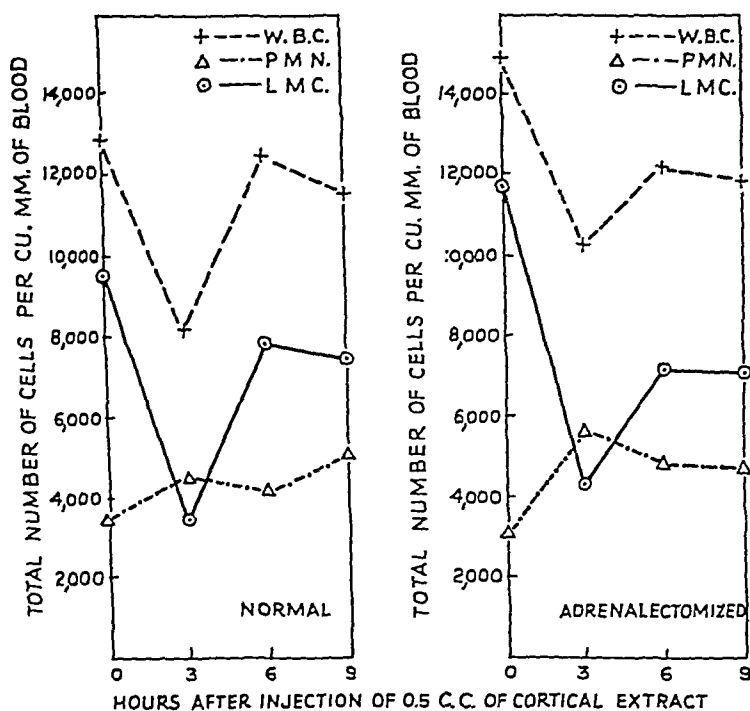


Fig. 8.—Effect of subcutaneous injection of 0.5 c.c. aqueous adrenal cortical extract on the leucocyte count of normal and adrenalectomized mice. There are eight animals at each interval in the unoperated series and six animals at each interval in the adrenalectomized group.

*Lymphopenic Response Produced by Various Adrenal Cortical Preparations.*—The role of the adrenal cortex as a mediator in the lymphopenic effect of adrenotrophic hormone led to a study of various adrenal cortical hormone preparations. It was of considerable interest to determine which adrenal cortical steroids might be primarily responsible for the lymphopenia. Single injections of aqueous adrenal cortical extract (Wilson) or of adrenal cortical steroids in oil (Upjohn) produced lymphopenic responses in normal and adrenalectomized mice and rats<sup>18</sup> (Fig. 8 and Table III). These same adrenal cortical preparations were also effective in rabbits and in man. Corticosterone

and compound E (Kendall) produced lymphopenia in adrenalectomized mice (Table III). On the other hand, desoxycorticosterone acetate, in the doses used, did not produce lymphopenia in either normal or adrenalectomized mice (Table III). This lymphopenic effect of adrenotropin and of certain adrenal cortical preparations has now been confirmed by other investigators in rats,<sup>22, 23</sup> dogs,<sup>24</sup> and human beings.<sup>25, 26</sup>

TABLE III. AVERAGE ALTERATIONS IN BLOOD CONSTITUENTS FOLLOWING SINGLE, SUBCUTANEOUS INJECTIONS OF ADRENAL CORTICAL STEROIDS IN NORMAL AND IN ADRENALECTOMIZED MICE

PREPARATION AND ANIMAL USED	NUMBER OF ANIMALS	DOSE (MG.)	TIME AFTER INJECTION (HOURS)	POLYMORPHO-NUCLEAR LEUCO-CYTES		
				W.B.C.	LYMPHO-CYTES	
Desoxycorticosterone acetate in sesame oil injected into normal mice	99	0.1	0	12,814	9,854	3,257
	5		3	13,680	7,929	5,751
	3		6	16,600	9,339	7,260
	3		9	17,667	9,266	8,400
Desoxycorticosterone acetate in sesame oil injected into normal mice	3	0.25	0	12,814	9,854	3,257
	3		3	26,700	13,269	13,431
	3		6	28,500	13,190	15,310
	3		9	15,050	8,995	6,065
Desoxycorticosterone acetate in sesame oil injected into adrenalectomized mice	60	0.0	0	14,903	11,717	3,189
	3		3	13,833	9,133	4,693
	3		6	10,700	8,640	2,060
	3		9	18,375	14,591	3,784
Desoxycorticosterone acetate in sesame oil injected into adrenalectomized mice	60	2.5	0	14,903	11,717	3,189
	3		3	16,033	10,357	5,676
	3		6	16,100	10,280	5,820
	3		9	13,517	10,277	3,263
Adrenal cortical steroids in oil injected into normal mice	99	0.1	0	12,814	9,854	3,257
	7		3	18,093	3,158	4,935
	3		6	16,933	10,088	8,845
	3		9	16,633	10,680	5,953
Adrenal cortical steroids in oil injected into adrenalectomized mice	60	0.1	0	14,903	11,717	3,189
	9		3	11,617	7,430	4,292
	3		6	13,617	12,157	1,460
	3		9	20,000	17,323	2,677
Compound F (Wintersteiner) injected into aqueous solution into adrenalectomized mice	60	0.25	0	14,903	11,717	3,189
	6		3	24,567	14,009	10,560
	6		6	14,767	6,733	8,034
	6		9	11,040	7,282	3,756
Corticosterone injected in aqueous solution into adrenalectomized mice	3	0.25	24	14,488	10,030	4,387
	60		0	14,903	11,717	3,189
	6		3	20,575	11,037	9,638
	6		6	15,400	8,050	7,356
	6		9	16,000	11,172	4,827
	3		24	16,767	10,560	6,207

*Pituitary-Adrenal Cortical Mediation of the Lymphopenia Induced by Stresses.*—A wide variety of physical, chemical, and physiologic stresses have long been known to produce a lymphopenic effect. The literature pertaining to this subject will be discussed subsequently. It was considered possible that some of these stresses might cause a lymphopenia as a result of pituitary-adrenal cortical stimulation. Data have been obtained confirming this hypothesis. The following types of stresses have been studied: a single injection of arsenite, benzene, histamine, pitressin, or typhoid vaccine; exposure to cold; and inanition. When exposed to any of these stresses, normal mice or rats re-

sponded with a lymphopenia, whereas adrenalectomized mice or rats showed no decrease in lymphocyte counts. Moreover, neither pitressin nor benzene induced lymphopenia in hypophysectomized rats. Some of these findings are shown in Figs. 9 and 10.

*Alterations Seen in Numbers of Polymorphonuclear Leucocytes.*—The term polymorphonuclear leucocyte is used here as a matter of convenience. The segmented forms should probably be referred to as heterophils in the mouse and the rat and as pseudoeosinophils in the rabbit. Other polymorphonuclear forms such as the basophil and the eosinophil are not grouped separately since they were few in number.

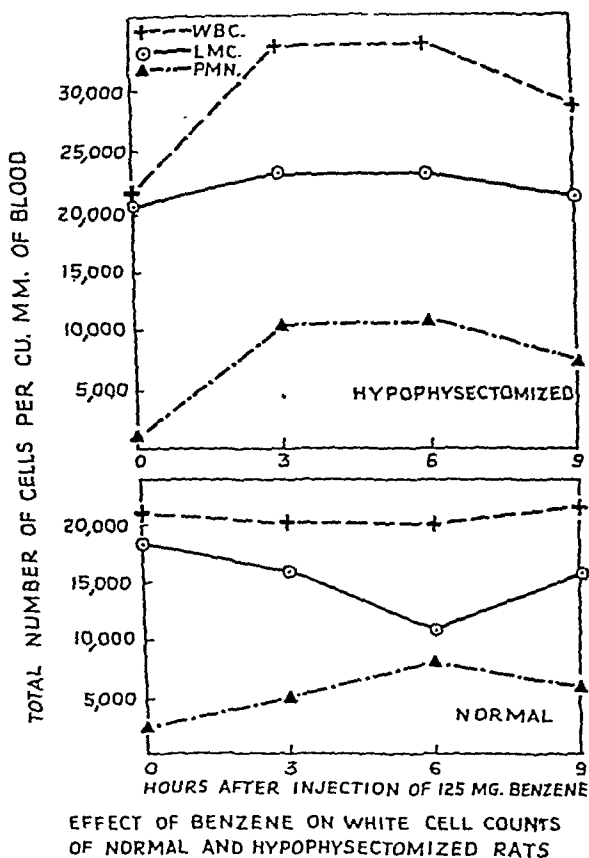


Fig. 9.—Effect of subcutaneous injection of 125 mg. benzene on the leucocyte counts of normal and hypophysectomized rats. Averages represent four animals at each interval in the hypophysectomized series and six animals in the normal series.

A slight but definite increase in the number of polymorphonuclear leucocytes was found in the mice given repeated injections of adrenotrophic hormone. It is questionable whether this increase was specifically related to the injection of the hormone, since a single injection of many agents, including adrenotrophin, produces an increase in circulating polymorphonuclear leucocytes when administered to either normal, adrenalectomized, or hypophysectomized animals (Figs. 7 to 10). The number of polymorphonuclear cells was lower, but not signifi-

cantly so, in the adrenalectomized mice. These cells were, however, increased in number in the adrenalectomized rats. In view of the fact that the polymorphonuclears did not increase in the blood of adrenalectomized mice, in spite of the hemoconcentration, it would seem that there was an actual suppression in the production or delivery of polymorphonuclears. A possible role of the adrenal cortex in the regulation of blood polymorphonuclear cells is suggested also by the work of Corey and Britton<sup>27</sup> who found a markedly decreased number of these cells in adrenalectomized cats. Pertinent discussion of this point has been presented previously.<sup>18</sup>

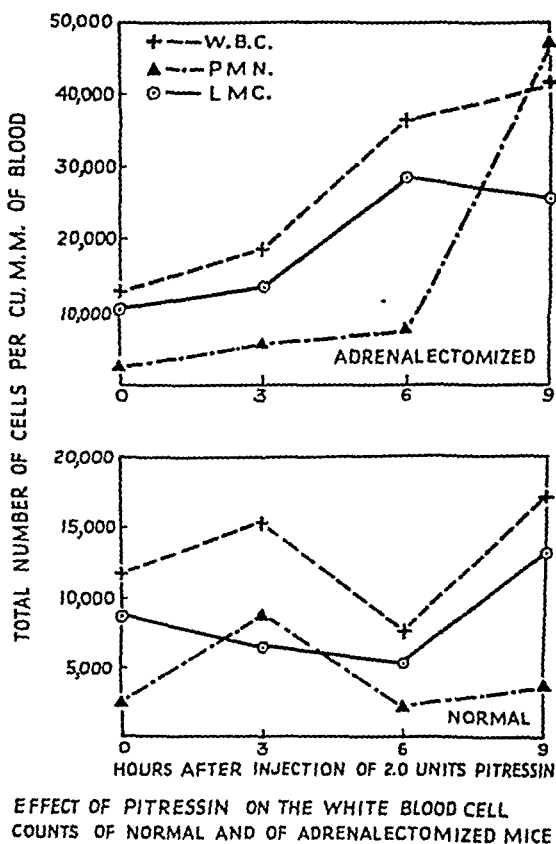


Fig. 10.—Alterations in numbers of leucocytes of adrenalectomized and normal mice following the subcutaneous injection of 2.0 units of pitressin. The control values are averages of ninety-nine mice in the unoperated group and forty-two in the adrenalectomized series. The values at each successive time interval were obtained on groups of six animals.

*Pituitary-Adrenal Cortical Control of the Numbers of Circulating Erythrocytes.*—Repeated injections of adrenotrophic hormone into normal mice resulted in a slight, but significant, polycythemia.<sup>20</sup> On the other hand, adrenalectomized mice, showing some degree of hemoconcentration despite daily injections of desoxycorticosterone acetate, had normal numbers of erythrocytes. Further, the significant decrease of red cells in adrenalectomized rats maintained with a normal whole blood specific gravity is additional evidence that pituitary-

adrenal cortical secretion exerts an influence over the numbers of circulating erythrocytes. The values for hemoglobin concentrations support these conclusions (Tables I and II).

In mice and rats given single injections of adrenotrophin, an initial increase in both hemoglobin concentration and red cell count was observed three hours after injection.<sup>15</sup> Thereafter, these constituents of the blood gradually declined, and twenty-four hours after hormone injection the red cells and hemoglobin concentrations were distinctly lower than normal. The specific gravity of the whole blood was not significantly altered during the twenty-four-hour period of study, although at the twenty-fourth hour there was evidence of a slight hemodilution.

Adrenal cortical preparations which produced a lymphopenia increased red cell counts and hemoglobin concentrations in a manner similar to that described for adrenotrophic hormone.

*Histologic Changes in Lymphoid Tissue Produced by Adrenal Cortical Secretion.*—The marked lymphopenia observed shortly following the injection of adrenotrophic or adrenal cortical hormones, or following a variety of stresses known to augment pituitary-adrenal cortical secretion, raised the problem of the mechanism inducing this lymphopenia. Several possibilities suggested themselves: failure of delivery of lymphocytes to the circulation, failure of lymphocyte production, or increased rate of peripheral removal of lymphocytes. The basis for the lymphopenia was sought in a detailed study of the lymphoid tissues following hormone administration.

In all the lymphoid tissues studied, that is, thymus, lymph nodes, spleen, and Peyer's patches, a single injection of adrenotrophic or of adrenal cortical hormones resulted in early dissolution of medium-sized and small lymphocytes within the lymphoid tissues. The dissolution of lymphocytes was accompanied by a marked edema. The term dissolution of lymphocytes has been used in the sense of a disintegration characterized by karyorrhexis pycnosis, or by a shedding of the cytoplasm of the cell.<sup>16, 17</sup> Downey and Weidenreich<sup>28</sup> have shown that under normal conditions lymphocytes may bud off portions of their cytoplasm. All of these alterations may be seen in the lymphoid tissues at the time of dissolution of lymphocytes. Certainly, the pycnosis and the disintegration of the nuclei of the lymphocytes result in death of these cells. However, many of the cells have markedly shrunken nuclei which have lost their normal chromatin distribution and have assumed various bizarre shapes. Although the cytoplasm of many of these cells has been lost, it is not to be construed that these cells are necessarily nonliving. It is possible that cells of this type might regenerate cytoplasm and again become normal lymphocytes.

In addition to the alterations in individual lymphocytes, there was an increased development of immature plasma cells similar to the cells described by Rohrer<sup>29</sup> or the "acute splenic tumor cell" of Rich, Lewis, and Wintrobe.<sup>30</sup> Investigations in our laboratory have demonstrated<sup>31</sup> that the increase in these cells occurs within the first few hours following hormone injection in rabbits. It has also been observed that not only is there an increased number of so-called

immature plasma cells, but that many smaller lymphocytes may develop basophilic cytoplasm, thus giving to these cells the superficial appearance of plasma cells.

The dissolution of lymphocytes is maximal between one and six hours after hormone injection in both mice and rabbits. This is then followed by a removal of cellular debris by macrophages. These macrophages are numerous in all of the lymphoid organs studied and contained large amounts of nuclear debris. Concomitant with the dissolution of lymphocytes, early alterations in fixed reticulum cells are evident. These changes consist in an increase in the amount and structure of cytoplasm of the histiocytes. Although these cells were derived from fixed reticulum cells, they are not highly active in removal of the vast amount of nuclear debris.

Within six to nine hours after administration of either adrenotrophic or adrenal cortical hormones, most of the nuclear fragments scattered about in the lymphoid tissues had been phagocytized, and the tissues in most of the animals at this time showed little evidence of further lymphocyte dissolution. During this period and subsequently the edema subsided and there was evident a beginning return to normal lymphoid structure. The period of recovery was characterized by differentiation of lymphocytes from reticulum cells and resumption of mitosis in lymphocytes. Within twenty-four hours after hormone injection, the lymphoid structures, with the exception of the thymus, contained normal numbers of lymphocytes. These described changes in lymphoid structures as a result of increased concentration of circulating adrenal cortical hormone have been confirmed by Yoffey.<sup>23</sup>

The high degree of specificity of the acute action of adrenal cortical steroids on the lymphocytes of animals is seen from the fact that bone marrow elements other than lymphocytes showed no evidence of degeneration. *Thus, lymphocytes represent an important site of action of certain adrenal cortical steroids.*

Examination of other tissues such as the lung showed that the lymphopenia was not due to an accumulation of the lymphocytes in any of the large capillary beds. None of the other tissues examined were edematous.

Adrenotrophic hormone injected into adrenalectomized mice had no effect on lymphoid tissue. Adrenal cortical extracts (aqueous or oil), corticosterone, or compound E when injected into adrenalectomized mice produced histologic alterations indistinguishable from those seen after administration of these preparations to normal animals. On the other hand, desoxycorticosterone acetate injection into normal or adrenalectomized mice had no effect on lymphoid tissue structure in the doses employed (0.025 to 2.5 mg.). Recently, it has been reported<sup>22</sup> that large doses of desoxycorticosterone acetate produced an atrophy of the thymus, but not of other lymphoid structures, in adrenalectomized rats.

A more detailed description of the histologic alterations resulting from increased concentrations of circulating adrenal cortical steroids has been published.<sup>17</sup> Any one of the nonspecific stimuli studied, and mentioned previously in connection with alterations in numbers of blood lymphocytes, induced alterations in lymphoid tissue in normal mice similar to those described for adreno-

trophic hormone or for the effective adrenal cortical preparations. However, these same stimuli, in the degree employed, had no effect on the lymphoid tissue of adrenalectomized mice.

*Adrenal Cortical Control of Release of Blood Globulin From Lymphocytes.*—The dissolution of lymphocytes observed following injection of adrenotrophic or adrenal cortical hormones suggested that a portion of the released cytoplasmic nitrogen could enter the metabolic pool and participate in physiologic processes involving nitrogen. A portion of the nitrogen released by lymphocyte dissolution was sought in the serum proteins. Since lymphoid tissue has been suggested as a site of antibody production (for a review of the literature see Perla and Marmorston<sup>1</sup>), examination of the globulin fraction of the serum was of particular interest.

The daily injection of 1 mg. of adrenotrophic hormone into normal mice for fifteen days produced a statistically significant increase in the total serum proteins. On the other hand, adrenalectomy in the mouse was followed by a significant decrease in the level of total serum proteins. This decrease was even more significant when considered in the light of the slight degree of hemoconcentration which occurred in the adrenalectomized mice despite the daily administration of small amounts of desoxycorticosterone acetate. A single injection of 3.0 mg. of adrenotrophin into rats resulted in a significant increase in the total serum proteins at three-hour and six-hour periods after hormone injection at which the sera were studied. At twenty-four hours after hormone, the level of serum proteins had returned to normal values.<sup>16, 33, 34</sup>

The injection of either adrenotrophic hormone or adrenal cortical steroids into normal rabbits produced a significant increase in the total globulin content of the serum during the three to twenty-four hour period that the animals were studied. Electrophoretic patterns revealed that the increase in globulin was a result of rises in both the beta and gamma globulin components of the rabbits' sera.<sup>33, 34</sup> The albumin and the alpha globulin fractions were unaltered. These data assume added significance in the light of the demonstration that washed lymphocytes from the lymphoid tissue of normal rabbits contain a protein which has an electrophoretic mobility identical with that of the normal gamma globulin of rabbits' blood.<sup>33, 34</sup> Kass,<sup>35</sup> working with human lymphoid tissue, has confirmed the presence in lymphocytes of a protein immunologically identical with normal serum gamma globulin.

A second component of lymphocyte extracts is probably identical with the beta globulin of rabbits' blood. It is, therefore, established that normal lymphocytes contain at least one, and possibly two, proteins which are indistinguishable from certain of the normal globulins of the blood, and that these proteins are released from lymphocytes to the circulation under circumstances of augmented pituitary-adrenal cortical secretion.<sup>34-36</sup>

*Adrenal Cortical Control of the Release of Antibodies From Lymphocytes.*—The demonstrated release of globulin from lymphocytes by adrenotrophic and adrenal cortical hormones suggested that these hormones might stimulate an increased production of antibodies in immunized animals. The daily administration of adrenal cortical extract to mice, rats, and rabbits being immunized



with a variety of antigens produced a higher level of antibody in the sera of those animals injected with hormone as compared to the sera of animals receiving antigen alone.<sup>37, 38</sup>

Because antibodies have been demonstrated in lymphocytes,<sup>39, 40</sup> augmented dissolution of these cells by adrenal cortical steroids would be expected to increase titer. In hyperimmunized rabbits a single injection of adrenal cortical extract (aqueous or oil), or of adrenotrophic hormone, resulted within six hours in a marked rise in antibodies in the blood.<sup>37, 38</sup> The maximum effect was observed at nine hours, following which time the titers gradually returned approximately to their previous levels. The data establish that higher titers of antibody appear in the immunized animal at a time after hormone injection when the levels of normal serum globulins are increased in nonimmunized animals. Serum protein alterations are maximum at the time that the greatest amount of lymphocyte dissolution is occurring and when the most striking lymphopenia is present in the blood. Extension of these experiments indicates that the anamnestic response is probably due to the pituitary-adrenal cortical control of the release of antibody from lymphocytes.<sup>36</sup> This hypothesis would explain why various nonspecific agents produce an anamnestic response<sup>41</sup> and would correlate this response with the simultaneously occurring lymphopenia.

#### SIGNIFICANCE OF RESULTS

The acute, absolute lymphopenia observed within a few hours after administration of adrenotrophic hormone is the peripheral manifestation of a sudden release of hormones of the adrenal cortex. This is evidenced by depletion of adrenal cholesterol and sudanophilic material shortly after adrenotrophic hormone injection. In addition, the lymphopenia reflects the marked lymphocyte dissolution occurring concomitantly in lymphoid structures and is, therefore, a result of the failure of delivery of normal numbers of lymphocytes to the circulation. Support for this point of view has been obtained in the experiments of Reinhardt and Li<sup>22</sup> who observed a 50 per cent decline in the numbers of lymphocytes in the thoracic duct lymph of the rat within thirty minutes following injection of adrenotrophic hormone. It should be noted that peripheral factors may also be concerned in the removal of lymphocytes, thus contributing to the lymphopenia.<sup>42</sup>

The lymphopenia and augmented serum globulin levels occur at a time when there is maximum dissolution of lymphocytes in the lymphoid structures (six to nine hours). Following this time, restoration of normal lymphoid structure is evident, lymphocyte dissolution ceases, and blood lymphocytes and serum protein values begin to return to normal. At this time there is also a beginning restoration of the adrenal lipids. Within a relatively few hours, therefore, profound physiologic alterations occur as a consequence of a single stimulation of the adrenal cortex and the effects of adrenal cortical steroids on the structure and function of lymphoid tissue. The rate of these physiologic processes is normally under pituitary-adrenal cortical control. This conclusion is further supported by the fact that in adrenalectomized animals there is a lymphocytosis,<sup>20, 34</sup> a diminished rate of lymphocyte dissolution,<sup>43</sup> and a lower

than normal total serum protein level.<sup>16, 33</sup> It should be emphasized that the endocrine mechanism is concerned with regulation of the rates of the processes which have been discussed. It is not to be construed, however, that these physiologic processes cease in the absence of the pituitary or the adrenals. In this respect the physiologic reactions influencing lymphoid tissue structure and function resemble other endocrine-controlled phenomena.

Inasmuch as a variety of unrelated stimuli are known to augment pituitary-adrenal cortical secretion, it might be expected that single exposures of an animal to any one of these stimuli would initiate and reproduce the sequence of alterations which have been described previously. Thus, shortly after the initiation of an infection there is adrenal cortical stimulation,<sup>19, 44-46</sup> an absolute lymphopenia,<sup>47, 48</sup> lymphocyte dissolution and reticulo-endothelial activation,<sup>48, 49</sup> and an increase in the serum globulins.<sup>50-54</sup> A single injection of a variety of toxic chemical agents, such as, arsenic, benzene, antimony, formaldehyde, colchicine, and foreign proteins, activates the adrenal cortex,<sup>19, 43</sup> induces a lymphopenia,<sup>55-59</sup> lymphocytic degeneration,<sup>7, 43, 60-63</sup> and augmented serum globulin levels.<sup>43, 64-66</sup> Anesthetics also produce a portion of this sequence of events.<sup>67, 68</sup> Radioactive agents, such as, x-rays, radium, and thorium X, are also effective in causing these alterations.<sup>42, 69-76</sup> Environmental stresses, such as, cold, heat, inanition, trauma, and anoxia, have been shown to reproduce one or more of the series of events under discussion.<sup>7, 19, 43, 77-84</sup> Finally, physiologic agents normally present in the organism, such as, insulin, epinephrine, histamine, pitressin, and thyroid hormone, may, when administered or when present in excess, augment adrenal cortical activity,<sup>19, 43</sup> induce a lymphopenia,<sup>43, 55, 57, 85-87</sup> cause lymphoid tissue dissolution,<sup>7, 10, 43, 87</sup> and in some instances cause an increase in serum globulins.<sup>43, 88</sup>

Most of the many kinds of stimuli previously mentioned have been shown to cause adrenal cortical activation, as demonstrated by histologic or chemical study of the adrenal. Moreover, some of these stimuli are without effect on lymphoid tissue in adrenalectomized animals.<sup>10, 17, 42, 43, 62, 84, 89</sup> The activation of the adrenal cortex is dependent upon the secretion of pituitary adrenotrophic hormone, since certain of these so-called nonspecific stimuli fail to produce any alterations in lymphoid tissue physiology in hypophysectomized animals. It is suggested that a variety of nonspecific influences which have been known for many years to affect lymphoid tissue may be exerting their action through pituitary-adrenal cortical activation.

Many nonspecific stresses may, when applied in large amounts or in great degree, affect lymphoid tissue without hormonal intervention. Thus, it has been shown that high concentrations of benzene will destroy lymphocytes in vitro.<sup>63</sup> The concentration of benzene required, however, was considerably higher than that which could be obtained in the living organism without producing death. Therefore, the dissolution of lymphocytes following benzene injection could not be accounted for on the basis of a direct action of the chemical on lymphocytes. However, the concentration of benzene which is required to produce lymphocyte dissolution in vivo is considerably less than that which will produce similar results in vitro. It is recognized that agents less toxic

systemically than benzene may have a direct destructive action on lymphocytes when administered to experimental animals. Agents of this type which have been shown to be effective in causing thymic involution in adrenalectomized animals when given in large, nonphysiologic doses are desoxycorticosterone, testosterone, estrogens, and diethylstilbesterol.<sup>10, 32, 30</sup> It should be emphasized that most of the data obtained with the use of nonspecific stimuli in relation to lymphoid tissue physiology support the hypothesis that pituitary-adrenal cortical mediation is involved.

A striking example of a stimulus which may exhibit either a direct or an adrenal cortical-mediated effect on lymphoid tissue may be seen from studies with x-rays.<sup>76, 91</sup> The long-recognized effect of x-rays on lymphoid tissue is, in the case of large doses, a direct action on lymphoid tissue, while in small doses, the lymphocytolytic effect of radiation is dependent on pituitary-adrenal cortical mediation.<sup>42, 89</sup>

In 1937, Selye<sup>7</sup> described certain responses, such as, depletion of adrenal cortical lipids, thymic atrophy, gastric hemorrhage, and clouding of the cornea, in experimental animals exposed to a variety of stresses. These reactions were grouped into a syndrome which was termed the "alarm reaction." Selye also noted that repeated exposure to increasing degrees of stress produced an increased degree of resistance against the stress; this phenomenon was called the "adaptation syndrome." The role of the adrenal cortex in the "alarm reaction" was suggested by Selye to be one of protection against histamine which was liberated in augmented amounts during the "alarm reaction." The involution of lymphoid tissue in the stressed animal has now been demonstrated to be due to the augmented secretion of pituitary adrenotrophic hormone.<sup>19</sup> As a result, there occurs an increased release of the steroid hormones of the adrenal cortex, which produce an atrophy of lymphoid tissue.<sup>11, 15, 18</sup> This atrophy is a consequence of the dissolution of lymphocytes which was described previously.

The extensive literature on the role of lymphoid tissue in resistance led us to suggest<sup>33</sup> that the pituitary-adrenal cortical control of lymphoid tissue structure and function integrated the role of the adrenal cortex and the lymphocyte in resistance. One important manifestation of this relationship is the augmented release of antibody from lymphocytes in circumstances of stress.<sup>36, 42</sup> The manner in which various factors play a role in resistance and adaptation is not well understood except in the case of the immune globulins.

Although many of the nonspecific stimuli which have been discussed produce an acute lymphopenia, the blood picture may change subsequently to one of lymphocytosis. Acute lymphocyte dissolution and lymphopenia are shortly followed by restoration of normal lymphocyte numbers in lymphoid structures. The duration of the lymphopenia may be dependent upon the degree to which the adrenal steroids are released, the period over which this accelerated release continues, and the rate of operation of processes concerned with the formation of lymphocytes. Various nonspecific stimuli do not affect the pituitary-adrenal cortical mechanism to the same degree, and, therefore, several types of physiologic manifestations of adrenal cortical activation may be seen.<sup>19</sup> In this con-

nection particular attention must be paid to the role of inanition in effecting involution of lymphoid tissue. Caloric restriction is a potent activator of pituitary-adrenal cortical secretion.<sup>19</sup> The effects of inanition on lymphoid tissue have been shown to be mediated primarily by this endocrine mechanism.<sup>84</sup> In addition, dietary intake will control the supply of materials from which new lymphoid tissue can be formed. It is particularly important to evaluate the inanition factor in nutrition studies designed to elucidate the role of a specific dietary constituent in the growth of lymphoid tissue.

Lymphocyte dissolution may be one of the physiologic stimuli which calls into play factors concerned with the synthesis of lymphocytes. These factors as yet have not been studied adequately. The numbers of circulating lymphocytes in the blood at any time must be a reflection of the balance obtaining among lymphocyte production, lymphocyte dissolution, and peripheral removal of lymphocytes. When the acute effects of the sudden release of adrenal cortical steroids have subsided, the mechanisms concerned with lymphocyte production may manifest themselves to an exaggerated degree. Therefore, the blood picture may alter from lymphopenia to lymphocytosis. The initial lymphopenia produced by most of the nonspecific agents which have been referred to in the previous paragraphs produce lymphocyte dissolution. Therefore, this acute lymphopenia is due to failure of delivery of lymphocytes to the circulation. The subsequent enhanced production of lymphocytes would explain the high level of lymphocytes, following an immediate lymphopenia, which occurs following insulin<sup>87</sup> or epinephrine injection,<sup>88</sup> after low doses of x-rays,<sup>73</sup> and the lymphocytosis in the recovery phase of acute infectious diseases.<sup>92, 93</sup> Blood lymphocyte pictures in disease may be better understood when interpreted in the light of the demonstrated function of lymphoid tissue and its peripheral manifestation, the numbers of circulating lymphocytes.

Many of the same stimuli which have been previously cited as augmentors of pituitary-adrenal cortical secretion, of lymphoid tissue dissolution, of the degree of lymphopenia, and of serum protein levels are also known to increase antibody titers in immunized animals.<sup>1, 41</sup> This enhancement of titer by non-specific stimuli is known as the anamnestic reaction. These relationships suggested that the anamnestic response was dependent upon the ability of these agents to increase adrenal cortical hormone secretion. Data obtained<sup>36, 42, 89</sup> establish clearly that an enhancement of antibody titer occurred when adrenal cortical preparations were administered to previously immunized animals having no circulating antibody. Adrenotrophic hormone was highly active in the normal but not in the adrenalectomized animal. Certain toxic agents, potassium arsenite, benzene, and low doses of x-rays produced the anamnestic reaction in normal animals. These agents were without effect in the absence of the adrenals. All of the adrenalectomized animals had demonstrable antibodies in extracts of lymphocytes from their lymphoid tissue. The pituitary-adrenal cortical mechanism is essential for the release of antibodies from lymphocytes except under those conditions in which there is a direct effect of the toxic agent on lymphocytes, such as high dose of x-rays in adrenalectomized animals.<sup>42, 89</sup>

The role of the adrenal cortex in controlling the rate of release of lymphocyte constituents correlates the functions of the adrenal and of the lymphocyte in resistance. The participation of the adrenal cortex is dependent upon its stimulation by pituitary adrenotrophic hormone.

#### CONCLUDING REMARKS

In the opening statements of this presentation, attention was called to the paucity of knowledge concerning the fate and function of the lymphocyte and the physiologic control of lymphoid tissue. One of the fates of the lymphocyte elements is a dissolution of many of these cells in the lymphoid structures. This dissolution often results in the disintegration and removal of the structural material of these cells. Some lymphocytes may shed portions of their cytoplasm into the surrounding lymph without death of the residual nuclei; under these circumstances, regeneration of normal amounts of cytoplasm may follow. The cytoplasm yielded to the lymph by the lymphocyte contains gamma globulin and, in the immunized animal, antibody. Gamma globulin is found within lymphoid structures, is present in lymphocytes, and its rate of release from these cells is under control of pituitary-adrenal cortical secretion. As a consequence of this hormonal control of the rate of lymphocyte dissolution, the numbers of lymphocytes in the blood and in lymphoid tissues is influenced by the degree of adrenal cortical activity.

#### REFERENCES

1. Perla, D., and Marmorston, J.: *Natural Resistance and Clinical Medicine*, Boston, 1941, Little, Brown & Company.
2. Drinker, C. K., and Yoffey, J. M.: *Lymphatics, Lymph, and Lymphoid Tissue*, Cambridge, 1941, Harvard University Press.
3. Bloom, W.: *Tissue Cultures of Blood and Blood-Forming Tissues*, in Downey, Hal (editor): *Handbook of Hematology*, New York, 1938, Paul B. Hoeber, Inc., vol. 2, sec. 20, p. 1471.
4. Kolouch, F., Jr.: *The Lymphocyte in Acute Inflammation*, *Am. J. Path.* 15: 413, 1939.
5. Grollman, A.: *The Adrenals*, Baltimore, 1936, Williams & Wilkins Company.
6. Moon, H. D.: Inhibition of Somatic Growth in Castrate Rats With Pituitary Extract, *Proc. Soc. Exper. Biol. & Med.* 37: 34, 1937.
7. Selye, H.: *Studies on Adaptation*, *Endocrinology* 21: 169, 1937.
8. Ingle, D. J.: Atrophy of the Thymus in Normal and Hypophysectomized Rats Following Administration of Cortin, *Proc. Soc. Exper. Biol. & Med.* 38: 443, 1938.
9. Ingle, D. J.: Effect of Two Steroid Compounds on Weight of Thymus of Adrenalectomized Rats, *Proc. Soc. Exper. Biol. & Med.* 44: 174, 1940.
10. Selye, H.: *Thymus Gland—The Alarm Reaction*, In the *Cyclopedia of Medicine, Surgery and Specialties*, Philadelphia, 1940, F. A. Davis Company, vol. 15, p. 15.
11. Reinhardt, W. O., and Holmes, R. O.: Thymus and Lymph Nodes Following Adrenalectomy and Maintenance With NaCl in the Rat, *Proc. Soc. Exper. Biol. & Med.* 45: 267, 1940.
12. Wells, B. B., and Kendall, E. C.: A Qualitative Difference in the Effect of Compounds Separated From the Adrenal Cortex on Distribution of Electrolytes and on Atrophy of the Adrenal and Thymus Glands of Rats, *Proc. Staff Meet., Mayo Clin.* 15: 133, 1940.
13. Sayers, G., White, A., and Long, C. N. H.: Preparation and Properties of Pituitary Adrenotropic Hormone, *J. Biol. Chem.* 149: 425, 1943.
14. Dougherty, T. F., and White, A.: Effect of Pituitary Adrenotropic Hormone on Lymphoid Tissue, *Proc. Soc. Exper. Biol. & Med.* 53: 132, 1943.
15. Simpson, M. E., Li, C. H., Reinhardt, W. O., and Evans, H. M.: Similarity of Response of Thymus and Lymph Nodes to Administration of Adrenocorticotrophic Hormone in the Rat, *Proc. Soc. Exper. Biol. & Med.* 54: 135, 1943.
16. White, A., and Dougherty, T. F.: Influence of Pituitary Adrenotropic Hormone on Lymphoid Tissue Structure in Relation to Serum Proteins, *Proc. Soc. Exper. Biol. & Med.* 56: 26, 1944.

17. Dougherty, T. F., and White, A.: Regulation of Functional Alterations in Lymphoid Tissue Induced by Adrenal Cortical Secretion, *Am. J. Anat.* 77: 81, 1945.
18. Dougherty, T. F., and White, A.: Influence of Hormones on Lymphoid Tissue Structure and Function. The Role of the Pituitary Adrenotrophic Hormone in the Regulation of the Lymphocytes and Other Cellular Elements of the Blood, *Endocrinology* 35: 1, 1944.
19. Sayers, G., Sayers, M. A., Fry, E. G., White, A., and Long, C. N. H.: The Effect of the Adrenotrophic Hormone of the Anterior Pituitary on the Cholesterol Content of the Adrenals, With a Review of the Literature of Adrenal Cholesterol, *Yale J. Biol. & Med.* 16: 361, 1944.
20. White, A., and Dougherty, T. F.: Effect of Prolonged Stimulation of the Adrenal Cortex and of Adrenalectomy on the Numbers of Circulating Erythrocytes and Lymphocytes, *Endocrinology* 36: 16, 1945.
21. White, A., Bonsnes, R. W., and Long, C. N. H.: Prolactin, *J. Biol. Chem.* 143: 447, 1942.
22. Reinhardt, W. O., and Li, C. H.: Depression of Lymphocyte Content of Thoracic Duct Lymph by Adrenocorticotrophic Hormone, *Science* 101: 360, 1945.
23. Yoffey, J. M.: Some Effects of Adrenotrophic Hormone (PATH), Extract of Suprarenal Cortex, and Colchicine on the Haemopoietic System, *J. Anat.* 80: 132, 1946.
24. Reinhardt, W. O., Aron, H., and Li, C. H.: Effect of Adrenocorticotrophic Hormone on Leucocyte Picture of Normal Rats and Dogs, *Proc. Soc. Exper. Biol. & Med.* 57: 19, 1944.
25. Darrow, D. C.: Personal communication.
26. Hellman, L.: Personal communication.
27. Corey, E. L., and Britton, S. W.: Blood-Cellular Changes in Adrenal Insufficiency and the Effects of Cortico-Adrenal Extract, *Am. J. Physiol.* 102: 699, 1932.
28. Downey, Hal, and Weidenreich, F.: Ueber die Bildung der Lymphocyten in Lymphdrüsen und Milz, *Arch. f. mikr. Anat.* 80: Abt. 1, 306, 1912.
29. Rohr, K.: Blut- und Knochenmarksmorphologie der Agranulocytosen, *Folia haemat.* 55: 305, 1936.
30. Rich, A. R., Lewis, M. R., and Wintrobe, M. M.: The Activity of the Lymphocyte in the Body's Reaction to Foreign Protein, as Established by the Identification of the Acute Splenic Tumor Cell, *Bull. Johns Hopkins Hosp.* 65: 311, 1939.
31. Dougherty, T. F., and White, A.: Increased "Plasma Cell" Production Following Adrenal Cortical Stimulation, *Anat. Rec. (Suppl.)* 94: 13, 1946.
32. Dontigny, P.: Morphologic Effect of Desoxycorticosterone Acetate on the Thymus, *Proc. Soc. Exper. Biol. & Med.* 63: 248, 1946.
33. White, A., and Dougherty, T. F.: The Pituitary Adrenotrophic Hormone Control of the Rate of Release of Serum Globulins From Lymphoid Tissue, *Endocrinology* 36: 207, 1945.
34. White, A., and Dougherty, T. F.: The Role of Lymphocytes in Normal and Immune Globulin Production, and the Mode of Release of Globulin From Lymphocytes, *Ann. New York Acad. Sc.* 46: 859, 1946.
35. Kass, E. H.: The Occurrence of Normal Serum Gamma-Globulin in Human Lymphocytes, *Science* 101: 337, 1945.
36. Dougherty, T. F., Chase, J. H., and White, A.: Pituitary-Adrenal Cortical Control of Antibody Release From Lymphocytes. An Explanation of the Anamnestic Response, *Proc. Soc. Exper. Biol. & Med.* 58: 135, 1945.
37. Dougherty, T. F., White, A., and Chase, J. H.: Relationship of the Effects of Adrenal Cortical Secretion on Lymphoid Tissue and on Antibody Titer, *Proc. Soc. Exper. Biol. & Med.* 56: 28, 1944.
38. Chase, J. H., White, A., and Dougherty, T. F.: The Enhancement of Circulating Antibody Concentration by Adrenal Cortical Hormones, *J. Immunol.* 52: 101, 1946.
39. Dougherty, T. F., Chase, J. H., and White, A.: The Demonstration of Antibodies in Lymphocytes, *Proc. Soc. Exper. Biol. & Med.* 57: 295, 1944.
40. Harris, T. N., Grimm, E., Mertens, E., and Ehrich, W. E.: The Role of the Lymphocytes in Antibody Formation, *J. Exper. Med.* 81: 73, 1945.
41. Cannon, P. R.: Antibody Production and the Anamnestic Reaction, *J. LAB. & CLIN. MED.* 28: 127, 1942.
42. Dougherty, T. F., and White, A.: Pituitary-Adrenal Cortical Control of Lymphocyte Structure and Function as Revealed by Experimental X-Radiation, *Endocrinology* 39: 370, 1946.
43. Unpublished observations.
44. Elliott, T. R.: Pathological Changes in the Adrenal Glands, *Quart. J. Med.* 8: 47, 1914.
45. Sperry, W. M., and Stoyanoff, V. A.: Effects of Long-Continued Cholesterol Feeding in Rats, *J. Nutrition* 9: 131, 1935.
46. Sarason, E. L.: Adrenal Cortex in Systemic Disease, *Arch. Int. Med.* 71: 702, 1943.
47. Pepper, O. H. P., and Miller, T. G.: The Relation of Allantoin Excretion to Leucopenia and Leucocytosis in Rabbits, *J. Infect. Dis.* 19: 694, 1916.

48. Conway, E. A.: Reaction of Lymphatic Tissue of Rabbits to Repeated Injections of Bacterium Monocytoenes, *J. Infect. Dis.* 64: 217, 1939.
49. Tuta, J. A.: A Study of the Lymphocytosis Following Intravenous Injections Into Rabbits of Suspensions and Extracts of *Hemophilus Pertussis*, *Folia haemat.* 57: 122, 1937.
50. Longworth, L. G.; Shedlovsky, T., and MacInnes, D. A.: Electrophoretic Patterns of Normal and Pathological Human Blood Serum and Plasma, *J. Exper. Med.* 70: 399, 1939.
51. Bing, J.: Further Investigations on Hyperglobulinemia (I), *Acta med. Scandinav.* 103: 547, 1940.
52. Cardon, L., and Atlas, D. H.: Incidence and Cause of Hyperproteinemia, *Arch. Int. Med.* 71: 377, 1943.
53. Kagan, B. M.: Hyperglobulinemia, *Am. J. M. Sc.* 206: 309, 1943.
54. Vassel, B., Partridge, R., and Crossley, M. L.: The Chemistry of Infectious Diseases. VI. Changes in the Blood Serum Proteins of Dogs During Type I Pneumococcal Pneumonia, *Arch. Biochem.* 1: 493, 1943.
55. Ruggeri, G.: The Influence of Histamine Hydrochloride on the Number of Leucocytes, *Boll. Soc. ital. biol. sper.* 4: 415, 1929.
56. Wallbach, G.: The Effects of Small Amounts of Thorium X and Benzene on Blood Lymphocytes, *Ztschr. f. d. ges. exper. Med.* 87: 340, 1933.
57. Moon, V. H., Lieber, M. M., and Kennedy, P. J.: Histamine and Leucocytosis, *Arch. Path.* 20: 209, 1935.
58. Halter, K.: Studies on the Leucocyte Count Upon the Administration of Inorganic Arsenicals, *Klin. Wchnschr.* 15: 52, 1936.
59. Dalton, A. J., and Selye, H.: The Blood Picture During the Alarm Reaction, *Folia Haemat.* 62: 397, 1939.
60. Bertschinger, D.: Experimentelle Untersuchungen über den Einfluss des Arsens auf den lymphatischen Apparat, *Folia haemat.* 57: 194, 1937.
61. Leblond, C. P., and Segal, G.: Action of Colchicine on the Adrenals and Lymphatic Organs, *Compt. rend. Soc. de biol.* 128: 995, 1938.
62. Leblond, C. P., and Segal, G.: Action of Toxic Agents on the Thymus of the Adrenalectomized Animal, *Compt. rend. Soc. de biol.* 129: 838, 1938.
63. Selling, L., and Osgood, E. E.: Action of Benzol, Roentgen Rays and Radioactive Substances on the Blood and Blood-Forming Tissues, In *Handbook of Hematology*, edited by Hal Downey, New York, 1938, Paul B. Hoeber, Inc., vol. 4, sec. 39, p. 2693.
64. Berger, W.: Hyperproteinemia Following Protein Injections, *Ztschr. f. d. ges. exper. Med.* 28: 1, 1922.
65. Breinl, F.: Beitrag zur Kenntnis der Serumweißkörper, *Arch. f. exper. Path. u. Pharmacol.* 65: 309, 1911.
66. Lang, S.: The Effect of Phenylhydrazine on Blood Proteins, *Ztschr. f. d. ges. exper. Med.* 80: 637, 1932.
67. Warner, D.: Effect of Sodium Amytal on Leucocytes of the Albino Rat, *Proc. Soc. Exper. Biol. & Med.* 33: 99, 1935.
68. Yoffey, J. M.: The Fall of the Blood Lymphocytes of the Dog Under Chloralose Anaesthesia, *J. Physiol.* 85: 450, 1935.
69. Mottram, J. C., and Russ, S.: Lymphopenia Following Exposures of Rats to Soft X-rays and the Beta Rays of Radium, *J. Exper. Med.* 34: 271, 1921.
70. Sturges, M. M., and Levin, I.: Influence of Radium and X-Rays on Frog Leucocytes, *Proc. Soc. Exper. Biol. & Med.* 18: 295, 1921.
71. Aubertin, C.: Treatment of Rheumatism by Thorium X Action on the White Blood Cells, *Bull. et mém. Soc. méd. d. hôp. de Par.* 38: 582, 1922.
72. Averseng, Delas, Jaloustre, and Maurin: Influence of Thorium X Upon the Blood Formula, *Comp. rend. Soc. de biol.* 178: 1321, 1924.
73. Murphy, J. B.: The Lymphocyte in Resistance to Tissue Grafting, Malignant Disease, and Tuberculous Infection, *Monographs of Rockefeller Inst. Med. Res.* No. 21, 1926.
74. Latta, J. S., and Ehlers, O. C.: The Effects on the Blood and the Hematopoietic Organs of Repeated Short Exposure to X-Rays, *Am. J. Anat.* 47: 447, 1931.
75. Henshaw, P. S.: Experimental Roentgen Injury. I. Effects on the Tissues and Blood of C<sub>H</sub> Mice Produced With Single Small Whole-Body Exposures, *J. Nat. Cancer Inst.* 4: 477, 1944.
76. Leblond, C. P., and Lacassagne, A.: Specific Action of Polonium on the Lymphatic System as Shown in Adrenalectomized Animals, *Am. J. Roentgenol.* 50: 801, 1943.
77. Murphy, J. B., and Sturm, E.: Effect of Dry Heat on the Blood Count in Animals. III. Studies on Lymphoid Activity, *J. Exper. Med.* 29: 1, 1919.
78. Jackson, C. M.: The Effects of Inanition and Malnutrition Upon Growth and Structure, Philadelphia, 1925, P. Blakiston's Son & Company.
79. Emmel, V. E., and Streicher, M. H.: Leucocyte Reaction to Acute Inanition, *Folia haemat.* 39: 223, 1929.

80. Meyer, O. O., Seevers, M. H., and Beatty, S. R.: The Effect of Reduced Atmospheric Pressure on the Leucocyte Count, *Am. J. Physiol.* 113: 166, 1935.
81. von Euler, H., and Malmberg, M.: The Blood Cell Constitution and Ascorbic Acid Contents of Guinea Pigs, *Ztschr. f. physiol. Chem.* 243: 121, 1936.
82. Shukers, C. F., and Day, P. L.: The Effects of Inanition and Riboflavin Deficiency Upon the Blood Picture of the Rat, *J. Nutrition* 25: 511, 1943.
83. Darrow, D. C., and Sarason, E. L.: Some Effects of Low Atmospheric Pressure on Rats, *J. Clin. Investigation* 23: 11, 1944.
84. Dougherty, T. F., and White, A.: Role of the Adrenal Cortex in Lymphoid Tissue Involution Produced by Inanition, *Anat. Rec.* 91: 7, 1945.
85. Latta, J. S., and Benner, M. C.: The Hemopoietic Disturbances Induced in the Albino Rat by Administration of Thyroxin, *Am. J. Anat.* 54: 115, 1934.
86. Garrey, W. E., and Bryan, W. R.: Variations in White Blood Cell Counts, *Physiol. Rev.* 15: 597, 1935.
87. Latta, J. S., and Henderson, J. W.: The Hemopoietic Disturbances Induced in the Albino Rat by Insulin Administration, *Folia haemat.* 57: 206, 1937.
88. Shirer, J. W.: A Study of Serum Protein in Hyperthyroidism, *Tr. Am. Ass. Study Goiter*, No. 89, 1932.
89. White, A., and Dougherty, T. F.: Significance of the Effects of X-Ray on Lymphoid Tissue, *Federation Proc.* 4: 109, 1945.
90. Gregoire, C.: Sur le mécanisme de l'atrophie thymique déclenchée par des hormones sexuelles, *Arch. internat. de pharmacodyn. et de therap.* 70: 45, 1945.
91. Leblond, C. P., and Segal, G.: Differentiation Between the Direct and Indirect Effects of Roentgen Rays Upon the Organs of Normal and Adrenalectomized Rats, *Am. J. Roentgenol.* 47: 302, 1942.
92. Longo, A.: Possibility of the Excitation of Leucopoiesis in Leishmanian Infection in Childhood, *Zentralbl. f. Biochem. Biophys.* 16: 121, 1914.
93. Reznikoff, P.: White Blood Cell Counts in Convalescence From Infectious Diseases, *Am. J. M. Sc.* 184: 167, 1932.



PURPURA FOLLOWING ESTROGEN THERAPY, WITH PARTICULAR  
REFERENCE TO HYPERSENSITIVITY TO (DIETHYL)  
STILBESTROL AND WITH A NOTE ON THE  
POSSIBLE RELATIONSHIP OF PURPURA  
TO ENDOGENOUS ESTROGENS

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THE literature indicates that the administration of estrogenic substances, and particularly stilbestrol, while at times productive of nausea and vomiting, does not cause serious toxicity in human beings. Thus, Shorr and co-workers,<sup>1</sup> Davis,<sup>2</sup> Davis and Boynton,<sup>3</sup> and MacBryde and associates<sup>4</sup> made careful observations of the blood in large series of women receiving 1 to 5 mg. of stilbestrol daily over long periods of time; they failed to find any significant changes in the blood or any evidence of a hemorrhagic tendency. The study of Karnaky<sup>5</sup> seems even more convincing in this respect. He gave remarkably large doses of stilbestrol to 136 women and eighty-six children. The adults received as much as 500 mg. at a single dose, 250 mg. once or twice weekly, or 100 mg. daily. The majority of the children were given 1 to 5 mg. daily; eight, however, received 10 to 25 mg. per day or every other day for periods of one to eight months. Karnaky relied on the sedimentation rate and complete blood counts to detect toxic effects. None were observed, and he therefore concluded that stilbestrol is entirely safe. Nevertheless, certain cases have been reported in the literature which indicate that occasional individuals may exhibit an idiosyncrasy or sensitivity to stilbestrol or other estrogenic substances, with resultant injury of the liver or bone marrow. Loftis<sup>6</sup> reported the case of a 44-year-old white woman who developed purpura over the lower extremities and bleeding from the gums while receiving weekly injections of an estrogen. The purpura disappeared within three weeks after the drug was stopped. Repeated trials with varying dosages always caused recurrence of purpura. Laboratory studies, including platelet counts, revealed nothing abnormal. A bone marrow examination was not reported. Chevalier and Umdenstock<sup>7</sup> observed a case of fatal myelophthisis following injections of large amounts of estradiol benzoate. This patient had received estradiol repeatedly over a period of ten years because of recurrent pruritus vulvae. The last course consisted of twenty injections of 10 mg. each in a two-week period. Shortly thereafter, the patient developed a profound anemia, leucopenia of 800 cells per cubic millimeter with 30 per cent neutrophils, and an outspoken purpura with 56,000 platelets per cubic millimeter. Herbst<sup>8</sup> observed purpura in two men receiving estradiol propionate for cancer of the prostate. There was no reduction of platelets in either case, and it was stated that the purpura was controlled by administration of calcium gluconate.

Instances of probable hepatotoxic effect of stilbestrol in human beings have been reported by Elias and Schwimmer<sup>9</sup> and Seligman.<sup>10</sup> The former case is of special interest in that the authors were able to demonstrate a distinct allergy to stilbestrol in their patient. They believed that the hepatocellular impairment was allergic in character. Jaundice was of relatively short duration and recovery was apparently complete.

A considerable literature has accumulated with respect to the effect of stilbestrol and other estrogens on the blood and blood-forming organs and the liver, in animal experiments. It is evident from these studies that there is a distinct difference in the sensitivity of different species. Arnold<sup>11</sup> showed that daily intramuscular injections of 4 to 10 mg. of stilbestrol in dogs commonly produced severe aplastic anemia and thrombocytopenic purpura. Tyslowitz and Dingemanse<sup>12</sup> found that dogs tolerated 1 mg. of estradiol or stilbestrol daily during a period of nine months without exhibiting any significant changes in the blood, but if given 5 mg. of either estrogen daily, a prompt fall in all blood elements occurred within one to three weeks after the injections were begun. The erythrocyte count and hemoglobin fell rapidly before any evidence of hemorrhage was noted. The platelets then dropped to very low levels and severe purpura occurred. The leucocytes showed an initial rise followed by a marked decrease in the number of granulocytes. Bone marrow studies in these dogs revealed a marked hyperplasia involving all of the cellular elements. These investigators<sup>12</sup> concluded that the estrogen caused a primary injury to the bone marrow followed by changes in the peripheral blood. Their results were confirmed by MacBryde and co-workers<sup>4</sup> and Crafts.<sup>13</sup> Castrodale and associates<sup>14</sup> compared the effect of stilbestrol and estradiol in dogs, using both parenteral and oral routes of administration, and found that thrombocytopenia and hemorrhage occurred sooner and that the survival time was shorter in those animals receiving estradiol. The onset of the blood changes and of purpura was delayed in the animals receiving the estrogen orally, but the severity was just as great after their appearance. They also noted that the bone marrow initially showed an increase of the myeloid elements with decreased areas of erythropoiesis, followed by a generalized hypoplasia. The megakaryocytes were markedly decreased early in the course of the experiment. The effect of stilbestrol in rats is evidently much less marked. A mild decrease in platelets early in the course of experiments in which large amounts of stilbestrol were injected was observed by von Haam and co-workers,<sup>15</sup> but there was no purpura nor any significant change in the leucocytes. The rat bone marrows, however, were markedly hyperplastic. Page and associates<sup>16</sup> implanted pellets of stilbestrol subcutaneously in rats and failed to notice any subsequent changes in the blood or bone marrow. Jacobson<sup>17</sup> injected relatively large doses of estradiol in white mice and found a moderate decrease in hemoglobin and red blood cells in the peripheral blood. He noted that the bone marrow of the mice showed a marked replacement of the marrow spaces by endostial new bone formation, the remaining areas of active marrow being hyperplastic. Crafts<sup>13</sup> and Tyslowitz and Hartman<sup>18</sup> were unable to produce any significant changes in the

blood or bone marrow of Rhesus monkeys, in spite of the administration of large amounts of estradiol or stilbestrol.

The effects of estrogens on the liver in animals has not been as extensively studied. Castrodale and associates<sup>14</sup> noted fatty metamorphosis and focal necrosis in the livers of many of their series of dogs receiving relatively large amounts of stilbestrol or estradiol, either orally or parenterally. Roberts and co-workers<sup>19</sup> later reported that large doses of estrone or estradiol benzoate produced changes in the liver, bone marrow, and peripheral blood of dogs, which were indistinguishable from those produced by stilbestrol. Hepatic changes consisted of fatty metamorphosis and hydropic degeneration.

It should be emphasized that in all of the experimental work which has been mentioned, the amounts of estrogenic substance necessary to produce evidence of injury were greatly in excess of the therapeutic dose in human beings. The purpose of the present report is to describe five cases in which purpura was first noted following estrogenic therapy. Four of these were in women, one was in a man. The latter individual also exhibited marked evidence of hepatic functional impairment.

#### CASE REPORTS

CASE 1.\*—E. S., a 55-year-old housewife, was first admitted to the University Hospital in March, 1943, because of weakness, menorrhagia, and purpura.

The patient had noted the onset of the menopause in the fall of 1936; it was characterized by hot flashes, increasing nervousness, weakness, and cessation of menses. In 1937 she consulted a local physician who gave her several intramuscular injections of amniotin in oil. She had little improvement of the symptoms and in June, 1939, consulted another physician. At this time intensive estrogen therapy was begun, and from June, 1939, until March, 1943, she received many intramuscular injections of estrone and estradiol. It has not been possible to determine the exact amount. In September, 1941, in addition, oral stilbestrol therapy was commenced. The patient received from 1 to 1.5 mg. of stilbestrol daily until March, 1943. In June, 1942, the patient was seen by the physician because of an episode of uterine bleeding which lasted three days. At this time she also complained of bruising very easily. The hemoglobin was found to be 80 per cent; the red blood cells were 4,000,000 per cubic millimeter; the leucocytes, 2,400 per cubic millimeter; and the platelets, 15,000 per cubic millimeter. Bleeding time by the Duke method was 15 minutes. The patient was treated with several blood transfusions, vitamin K, liver extract, and pectin, but moderate uterine bleeding lasted three to four days and recurred regularly each month. She continued to bruise easily on slight trauma.

In December, 1942, the patient first noticed petechiae over the legs, forearms, and chest. In February, 1943, there was a profuse uterine hemorrhage, and she was admitted to a private hospital. The examination of the blood at this time showed the hemoglobin to be 60 per cent; the red blood cells were 3,000,000 per cubic millimeter; the leucocytes, 2,900 per cubic millimeter; and the platelets, 8,700 per cubic millimeter. During this period of hospitalization the patient was given several blood transfusions and five deep x-ray treatments to the ovaries. She was discharged after nine hospital days. Uterine bleeding stopped on March 15, 1943, after four weeks of continuous rather severe hemorrhage, but the purpura persisted, and on March 25, 1943, she was admitted to the University of Minnesota Hospital. It should be noted that the patient had continued to take stilbestrol up to this time.

The past history revealed nothing of significance. The catamenia had been entirely normal prior to the menopause, and there was no previous indication of a hemorrhagic tendency.

\*This case was studied through the courtesy of Dr. H. W. Quist, Minneapolis, Minn.

Physical examination revealed numerous petechiae over the body and several large ecchymoses over the patient's legs, chest, and forearms. The spleen was barely palpable on deep inspiration. There were no other findings of significance.

The hemoglobin was 10.8 grams per 100 c.c.; the leucocytes were 2,000 per cubic millimeter; the reticulocytes, 1.5 per cent; and the platelets, 160,000 per cubic millimeter. The bleeding time by the Duke method was 42 minutes; the prothrombin time was normal. The cephalin-cholesterol flocculation test was 3 plus at 24 hours. A sternal bone marrow puncture revealed generalized hypoplasia of all the cellular elements of the bone marrow. Megakaryocytes were very scarce and showed degenerative changes.

All estrogenic therapy was stopped. The patient was given several blood transfusions, and was discharged in relatively good condition. It is noteworthy that she was complaining rather severely of hot flashes and that she wished very much to take additional stilbestrol for relief. She was advised strongly not to do this.

The purpura gradually disappeared, and the patient was relatively free of hemorrhages so far as can be determined from April, 1943, until July, 1944, when petechiae and ecchymoses again appeared over the legs, arms, and chest. It may be noted, however, that the platelets were repeatedly found to be reduced during this interval: 2,200 on April 16, 1943; 38,000 on Aug. 24, 1943; 6,400 on Oct. 18, 1943; and 148,000 on Feb. 10, 1944. The uterine bleeding did not recur. In September, 1944, she was given twelve roentgen treatments of ten minutes each to the ovaries, a total of 3,474 r.; the purpura persisted, however, and the patient was readmitted to the University Hospital in December, 1944. Physical examination at this time was essentially negative except for marked purpura over the legs, forearms, and chest. The hemoglobin was 11.5 grams; the leucocytes were 2,050 per cubic millimeter with 62 per cent neutrophils. The platelets were 62,000 per cubic millimeter. The prothrombin time was again normal and cephalin-cholesterol flocculation test at this time was negative. The bleeding time was markedly increased. Several sternal bone marrow punctures were attempted, but no marrow could be aspirated. After four days in the hospital, the patient was discharged and has been followed in the Out-patient Clinic until the present time. She has persisted in having purpura of moderate degree, and the platelets have varied on a number of occasions between 30,000 and 120,000 per cubic millimeter. The leucocytes have varied between 1,800 and 3,100 with 50 to 65 per cent neutrophils. The hemoglobin ranges between 10.5 and 12.5 grams per 100 c.c., and the red blood cells range between 3.8 and 4.5 million per cubic millimeter. Trial of various substances has been made, including refined liver extracts by intramuscular injection, whole desiccated liver by mouth, and folic acid by injection in amounts ranging up to 50 mg. per day. None of these has had any demonstrable effect.

This patient, then, has a chronic thrombocytopenia purpura and leucopenia, which is remarkably stable at a level permitting a reasonable degree of activity but which, nevertheless, interferes with her life to a considerable extent and threatens at any time to become more severe.

CASE 2.—Y. T., a 53-year-old housewife, was admitted to the University Hospital in December, 1944, because of purpura over the arms, legs, and chest.

The patient had the onset of the menopause in January, 1942, when the menses became irregular and profuse and she began to have increased nervousness, weakness, and hot flashes. In May, 1942, she had an episode of severe menorrhagia. Hysterectomy and bilateral salpingo-oophorectomy were performed at a private hospital, and at this time, May, 1942, oral stilbestrol therapy of one tablet daily was begun and continued until November, 1944. In November, after two and one-half years of continuous stilbestrol therapy, the patient first noticed petechiae and ecchymoses over the arms, legs, and chest. These persisted and she entered the University of Minnesota Hospital on Dec. 13, 1944. The past history revealed nothing of significance other than a mild arthritis of the cervical spine for which intravenous injections of streptococcus vaccine were given during October and November, 1944. This will be considered again in the following.

The physical examination revealed nothing of significance other than many petechiae and several ecchymoses over the legs, arms, and chest.

The hemoglobin was 10.3 grams; the red blood cells were 4,000,000 per cubic millimeter; the leucocytes, 5,250 with 57 per cent neutrophils; and the platelets, 70,000 per cubic millimeter. The cuff test was negative. The bleeding time by the Duke method was 16 minutes. A platelet count performed one week later was 90,000 per cubic millimeter. Stilbestrol therapy was stopped when the patient entered the hospital. The purpura rapidly improved, and after three days she was discharged to the Outpatient Clinic where she has been followed to the present time. Within two weeks from the time of discharge the petechiae had entirely disappeared. On Dec. 26, 1944, the platelet count was 180,000 per cubic millimeter; on Jan. 2, 1945, 210,000; and on March 14, 1945, 180,000. The sternal bone marrow obtained during the hospital admission revealed a marked hyperplasia involving chiefly the granulocytes. The megakaryocytes were also increased in number. The brom-sulfalein test (5 mg. per kilo at 45 minutes) was negative but the cephalin-cholesterol flocculation test was 3 plus in 48 hours. The urine urobilinogen was normal.

There was no recurrence of purpura until March 1, 1947. Shortly before this, the patient had taken benzestrol<sup>10</sup> because of persistent hot flashes. The onset of purpura was noted three days after the benzestrol was started. The drug was discontinued at once and after four days the purpura began to disappear. By March 25, it was no longer evident. On March 11 when it was still widespread, the platelets numbered 165,000 per cubic millimeter and the cuff test was strongly positive.

Streptococcus vaccine therapy was resumed in 1945 and has been continued irregularly until the present time. There has been no evidence of any recurrence of the purpura except on the one occasion following benzestrol. Thus, it appears improbable that the vaccine had any relation to the thrombocytopenia.

CASE 3.\*—S. G., a 23-year-old housewife, had been well except for irregularity of the menstrual periods. The interval had always been greater than thirty days, and since her marriage in 1943, the periods were even more irregular, there having been not more than eight or ten periods in the two years. On Feb. 15, 1945, because of not having had a period since October, 1944, the patient consulted a physician who gave her  $\frac{1}{2}$  c.c. benzestrol.<sup>10</sup> This dose was repeated on Feb. 21 and 22 and on March 6. On April 4, she was given 2.5 mg. of dimenformin benzoate, and on April 5, 12.5 mg. of trigestin. One week later menstruation commenced and lasted for five days. Two days after termination of the period a "rash broke out all over her body." She noticed that it was worse on the legs and hands but that it was present everywhere to at least a slight degree. She also noted a few bruises on various parts of the body.

The past history did not reveal anything of significance. The family history was of interest in that a sister died at the age of 23 of leucemia. The patient has two brothers and one sister living and well. Her parents are also well.

She has had occasional headaches, for which she takes a tablet composed of acetophenetidine, aspirin, and caffeine. She has taken one of these about every two weeks since October, 1944.

The physical examination revealed nothing other than a diffuse purpura. Multiple petechiae were present with increasing frequency toward the distal portions of the extremities, most marked on the lower legs and ankles. However, there were a few on the face, chest, abdomen, arms, hands, and thighs. The liver and spleen were not palpable.

The hemoglobin was 13.7 grams; the red cells were 4,150,000; the leucocytes, 7,200 per cubic millimeter with 61 per cent neutrophils; the platelets, 12,100; the bleeding time by the Duke method 24 minutes. A repeat platelet count was 20,000; the prothrombin was 100 per cent of normal. On April 25, there was no evidence of further spread of the purpura, but the platelets were only 11,000. On June 6, the platelet count was 13,200, the bleeding time 23 minutes. The bone marrow obtained by sternal puncture was said to reveal nothing abnormal. On July 27, 1945, the platelet count was 33,000, and on August 9, 40,000. At this time the purpura had almost entirely disappeared. On November 13, a few new petechiae

\*This case is reported through the courtesy of Dr. J. Garrott Allen of the Department of Surgery and the Metallurgical Laboratory, University of Chicago.

were observed. The irregular menstruation with long intervals between the periods has continued. The platelet count on Nov. 13, 1945, was 12,000. On Jan. 2, 1946, the patient was seen because of recurrence of petechiae in moderate number, especially on the legs. The patient was last observed on Feb. 16, 1946. At this time the hemoglobin was 10.9 grams; the red blood cells were 3,880,000; the leucocytes, 5,700 with 62 per cent neutrophiles; the platelets, 15,500. Only a few petechiae were noted.

CASE 4.\*—M. C. was a 79-year-old retired businessman. In 1939, at the time of transurethral resection, it was found that the patient had a carcinoma of the prostate. Stilbestrol was commenced on July 3, 1942, 0.5 mg. daily. From Nov. 17, 1943, until shortly before admission to the hospital, he took, somewhat intermittently, 3 mg. daily. The breasts had become distinctly enlarged. From time to time during this period the patient had received small amounts of sulfadiazine because of a urinary tract infection. He had not, however, received any sulfadiazine for three months prior to death and for one month prior to the first appearance of purpura. Ecchymoses first became evident on the extremities and neck about Feb. 25, 1946. Because of increasing purpura with some very large ecchymoses, particularly over the abdomen, the patient was admitted to the hospital on March 10, 1946. The hemorrhages were in the form of large ecchymoses and even considerable extravasations of blood under the skin. One massive extravasation was noted over the lower abdomen extending down into the pubic area and including the scrotum, also down onto the thighs. There were numerous ecchymoses elsewhere but small petechiae were very rare. There was no jaundice at the time of admission. The liver and spleen were not palpable, and there was no lymphadenopathy. There were no evidences of metastases to the bones. (X-rays of spine and pelvis were negative.) The platelet count was 40,000 on the day following admission. On the next day it was found to be 70,000. The prothrombin time was 17 per cent of normal. The serum albumin was 2.6 grams per cent; the serum globulin, 2.3. The cephalin-cholesterol flocculation test was 3 plus in 24 hours. The urine urobilinogen was 42 mg. in 24 hours (normally not above 3.5 mg.). The later course in the hospital was characterized by retinal hemorrhages, hemoptysis, and gastrointestinal bleeding, in addition to further bleeding into the skin. Coincident with transfusions and vitamin K, the prothrombin time came up to 80 per cent of normal, following which there was a temporary cessation of hemorrhages. Later, however, in spite of continued vitamin K administration, the prothrombin time dropped to 30 per cent and the generalized hemorrhagic tendency recurred. During the last six weeks in the hospital, the platelet count gradually rose to 150,000, but the bleeding persisted. An additional feature of interest that was not explained was a marked hypocalcemia with tetany. The serum calcium was 6.8, and on frequent occasions there was manifest tetany requiring injection of calcium. Death was believed to be due to anemia and exhaustion. Slight jaundice was observed in the last ten days of life. There was no evidence of steatorrhea.

Necropsy in this case revealed very little. The prostate was small and rather soft but exhibited definite adenocarcinoma microscopically. The liver was described as being "granular and firm," but not enlarged. Microscopically the sections of the liver revealed nothing other than mild passive congestion. The bone marrow was not examined.

CASE 5.†—H. G. was a 45-year-old housewife. Menstruation first became irregular in February, 1942. There was no period in May, 1942, and again from July to October, although during this interval one brief period of rather profuse bleeding for two hours occurred. In January, 1943, the patient was overemotional, had swelling of the hands, tingling of the skin, chills, sweats, and hot flashes. She was given 0.5 mg. of stilbestrol daily for one week, together with nembutal,  $\frac{1}{2}$  grain three times daily. She was seen two weeks later, at which time the nervousness and exhaustion, and also the hot flashes and sweats, were considerably improved. Stilbestrol in an amount of 0.5 mg. daily was continued for one week. From this time on the stilbestrol was given intermittently in the same dose for

\*This case was seen in consultation with Dr. D. P. Head to whom the authors acknowledge their gratitude for permission to report the findings which are given.

†This case was studied through the courtesy of Dr. Henry L. Ulrich, Minneapolis, Minn.

a period of four months. In August, 1944, when the patient was next seen, perspiration was marked and there were frequent dizzy spells, particularly on waking up in the morning. Stilbestrol was resumed in a dose of 0.2 mg. daily. She was not seen again until March, 1945. The same dose, namely, 0.2 mg., was prescribed again. The patient was also given calcium lactate, 5 grains three times a day, and  $\frac{1}{2}$  grain of nembutal three times daily. She was next seen in June, 1945, and stilbestrol was given again, 0.2 mg. daily. In August she was given 2,000 units of estrone hypodermically. This dose was repeated in November, 1945, the stilbestrol being taken intermittently in the interval. Bleeding was first noted in June, 1946. This was from the gums and uterus. From this time on there has been recurrent uterine bleeding, also intermittent hemorrhage from the gums and nose, and purpura of the skin. Numerous petechiae and ecchymoses have been noted on a number of occasions.

The physical examination revealed nothing of significance in other respects. The spleen and liver were not palpable and there was no lymphadenopathy.

Blood examination on June 6, 1946, revealed the following: Hemoglobin 80 per cent, red blood cells 4,430,000, leucocytes, 3,700, 49 per cent neutrophils, 36 per cent lymphocytes, 11 per cent monocytes, 3 per cent eosinophiles, and 1 per cent basophiles. On Sept. 24, 1946, the hemoglobin was 14.5 grams; the platelet count, 121,000 per cubic millimeter; the bleeding time, 5 minutes, 2 seconds (Duke method); the cuff test, markedly positive, 45 petechiae in a circle 1 inch in diameter on the forearm; the prothrombin time by the Quick method, 12.7 seconds (control, 12.4). Since this initial study, frequent platelet counts, bleeding times, and cuff tests have been carried out with essentially the same findings, regardless of the degree of purpura exhibited by the patient at the particular time. So far as is known she has not received any estrogen therapy since June, 1946. The purpura has not been alarming up to the present time, and the patient has been able to continue her activities on a limited scale.

#### STUDIES OF SKIN SENSITIVITY TO ESTROGENIC SUBSTANCES

Three of the previously described patients (Cases 1, 2, and 5) were available for skin tests to determine possible sensitivity to estrone and stilbestrol. Twenty-five individuals, either normal or suffering from various diseases, served as controls. Sixteen of these were tested for sensitivity to estrone, the remainder to stilbestrol. In each instance the test consisted of injecting intradermally 0.1 c.c. of a 0.2 per cent suspension in physiological saline. The same amount of the vehicle alone was injected in the opposite arm as a control. Observations of reactions were made at 30 minutes, 1 and 4 hours, and 24 hours, as noted in the following. The results of the tests are given in Table I. It is seen that Cases 1 and 2 exhibited distinct skin sensitivity to stilbestrol, while Cases 2 and 5 were sensitive to estrone. In none of these three cases was there any sensitivity to the vehicle. The reactions in each instance were of erythematous type, only slightly raised and without definite whealing. Their appearance suggested the presence of an active capillary or arteriolar hyperemia without transudation. Two of the control cases exhibited sensitivity to the estrone solution and one of these was positive to the solution of stilbestrol, but both showed equally positive reactions to the vehicle, so they cannot be classified as positive to the estrogens. Thus, it is evident that the only positive reactors among the twenty-eight individuals were the three with purpura, Cases 1, 2, and 5 of the present series. So far as could be determined from the records of the control individuals none had at any time received estrogen therapy.

Prausnitz-Küstner passive transfer was attempted in Cases 2 and 5 with estrone and in Cases 1 and 2 with stilbestrol, but this was uniformly negative.





Thus, it is questionable whether the skin reactions which were noted were due to circulating antibody. Precipitin tests were not carried out due to the insolubility of stilbestrol and estrone in water. The nature of these skin reactions is not understood but it seems likely that their significance is fundamentally related to the clinical manifestations in these cases, especially in view of the relatively large number of negative controls. The possibility of a specific tissue sensitivity is considered.

#### DISCUSSION

While the cases described in the foregoing cannot be accepted as proving a causal relationship between stilbestrol or other estrogenic substances and purpura, they do, nevertheless, suggest that such a relationship may at times exist, and they point to the need of greater conservatism in the administration of these substances. They also indicate the desirability of frequent observations with respect to the possible appearance of a hemorrhagic tendency in patients receiving continued estrogenic therapy. It is believed justified, in view of the present results of the skin sensitivity studies, to skin test patients in advance of the prescribing of estrogenic substances. In this connection, however, we recognize that the sensitivity shown by the present cases may have been acquired rather than inherent. In any event the appearance of signs of bone marrow or capillary injury, or of hepatic functional impairment, would appear to warrant discontinuance of estrogen therapy and the application of skin tests to determine possible sensitivity to estrogenic substances. It is clear that a relationship in these cases would have to be regarded as an idiosyncrasy or hypersensitivity, possibly analogous to the occasional case of neutropenia or agranulocytosis which follows amidopyrine, or the thrombocytopenic purpura at times seen following sedormid therapy, and concerning which a number of papers have appeared in the literature. Evidence as to the usually innocuous character of stilbestrol was cited at the outset. The onset of purpura following the exhibition of stilbestrol and its disappearance and reappearance after discontinuance and readministration, as in Case 2 of the present series and in the case reported by Loftis,<sup>6</sup> lend further support to the belief in a causal relationship. The regular production of thrombocytopenic purpura in dogs with amounts of stilbestrol larger than those commonly used in human therapy is quite compatible with the concept that the occasional cases of purpura noted in human beings after stilbestrol therapy represent idiosyncrasies or hypersensitivity to the drug. The possibility cannot be excluded that the purpura in Case 3 was related to the occasional ingestion of acetophenetidin.

It is also of interest that a number of papers have called attention to the association of purpura with menstruation.<sup>21-24, 26, 28-30</sup> In these studies it was at times noted that the platelet counts were normal between the periods, only to decline at the beginning of each menstruation and with the onset of purpura. With the cessation of menstruation, the platelet counts returned to normal and the purpura disappeared. Nagy<sup>22</sup> reported two cases of thrombocytopenic purpura in which irradiation of the ovaries was followed by an increase of platelets with disappearance of purpura and menorrhagia. For these instances,

Nagy suggested the term "purpura dysovarica." As a matter of fact, it was chiefly because of Nagy's report that radiation was given to the ovaries in Case 1 of the present series. No apparent benefit was noted, but it is quite likely that the bone marrow injury in this instance was exogenous while it would appear to have been endogenous in Nagy's cases. In this connection, attention may be called to a case reported by Leschke and Wittkower.<sup>24</sup> A 23-year-old woman had had severe menorrhagia since onset of the menses at the age of 11. When the patient reached the age of 22, the platelets were found to be 3700 per cubic millimeter. The surgeon removed the uterus and a cystic right ovary. This was followed by prompt cessation of the hemorrhagic tendency, and for two years there was no difficulty, after which time, however, the purpura recurred.

The relative frequency of thrombocytopenic purpura in women after puberty has been stressed repeatedly.<sup>26, 28</sup> Purpura is relatively common during pregnancy and has often constituted a severe complication. A number of the cases collected from the literature by Rushmore<sup>27</sup> exhibited purpura only during pregnancy, and some of them had a recurrence of purpura in subsequent pregnancies. On the other hand, Evans and Perry<sup>28</sup> observed a case in which a thrombocytopenic purpura disappeared entirely during pregnancy, only to reappear after delivery.

There is disagreement as to the platelet counts of normal women during their menstrual cycles, Genell<sup>31</sup> and Pohle<sup>32</sup> having reported a noticeable decrease in the platelet count at the beginning of each period and Damashek<sup>33</sup> having found just the opposite. David<sup>33</sup> emphasized the occurrence of hemorrhages and of positive Rumpel-Leede (capillary fragility) tests during menstruation. This question obviously deserves further study.

The possibility of sensitivity to endogenous estrogen, as suggested by the foregoing, is supported in some measure by the fact that two of the cases in the present series exhibited skin sensitivity to estrone, a natural estrogenic substance. One might assume that in certain individuals, a tissue, perhaps a capillary sensitivity, if not inherent, becomes established as time goes on. Evidence is lacking as to the nature of this sensitivity and the manner in which it might operate to produce purpura. The concept of sensitivity to endogenous estrogen has been elaborated by Zondek<sup>34, 35</sup> under the general appellation of "endocrine allergy." Zondek has demonstrated skin sensitivity to natural estrogens in a variety of pathologic states and has shown that desensitization is frequently beneficial.

The possibility of hepatotoxic effect of stilbestrol is suggested both by the animal experiments and the human cases previously mentioned.<sup>9, 10</sup> In Case 2 of the present group the cephalin-cholesterol flocculation test was positive in association with the thrombocytopenic purpura and negative after discontinuance of stilbestrol and disappearance of the purpura. The possibility exists, however, that there was a mild liver injury secondary to the purpura and unrelated to the stilbestrol. Case 4 of the present series was of particular interest because of the association of a severe hemorrhagic state with thrombocytopenia and hypoprothrombinemia, together with other evidence of liver

functional impairment following long continued use of stilbestrol for carcinoma of the prostate. The autopsy findings in this case did not indicate that the hemorrhagic state was related either to the carcinoma or to any demonstrable liver disease of ordinary type.

#### SUMMARY AND CONCLUSIONS

1. Five cases are reported in which purpura followed the administration of stilbestrol or other estrogenic substances. Four of these were in women being treated for menopausal symptoms or amenorrhea; one was in an elderly man with carcinoma of the prostate, without evidence of metastases.

2. In four of these cases the estrogen therapy, mainly stilbestrol, had been given for long periods, ranging from two and one-half to seven years. In one of these four, however, there were long intervals during a total of three years, in which none was given. In the fifth case estrogens were given parenterally, intermittently over a two-month period.

3. The purpose of reporting these five cases is simply to call attention to the possibility of a causal relationship between stilbestrol and other estrogens and purpura. Evidence of hypersensitivity to stilbestrol or estrone or both, was manifested in each of the three cases available for skin testing, although absent in twenty-five control individuals. Circulating antibody could not be demonstrated by passive transfer. Nevertheless, the possibility must be considered that hypersensitivity to exogenous or endogenous estrogens may at times constitute the major factor in the production of purpura.

4. The literature relating to estrogenic substances is discussed from the following standpoints: (a) The reported cases of supposed toxic effect in human beings, in which abnormalities related to the liver or blood were noted; (b) the experimental production of purpura and hepatic injury in animals by means of their administration; and (c) the question of effect of endogenous estrogen in the production of purpura.

#### REFERENCES

1. Shorr, E., Robinson, F. H., and Papanicolaou, G. N.: Clinical Study of Synthetic Estrogen Stilbestrol, *J. A. M. A.* 113: 2312, 1939.
2. Davis, M. E.: Clinical Study of Stilbestrol, *Am. J. Obst. & Gyn.* 39: 938, 1940.
3. Davis, M. E., and Boynton, M. W.: Indications, Clinical Use, and Toxicity of 4-4' Dihydroxydiethyl Stilbene, *J. Clin. Endocrinol.* 1: 339, 1941.
4. MacBryde, C., Freedman, H., Loeffel, E., and Castrodale, D.: Synthetic Estrogen Stilbestrol; Clinical and Experimental Studies, *J. A. M. A.* 115: 440, 1940.
5. Karnaky, K. J.: Prolonged Administration of Diethylstilbestrol, *J. Clin. Endocrinol.* 5: 279, 1945.
6. Loftis, E. L.: Purpura Due to Injection of Estrogenic Substance, *Arch. Dermat. & Syph.* 43: 138, 1940.
7. Chevalier, P., and Umdenstock, R.: Myelophthise, Mortelle Apris Injections De Benzoate D' Oestradiol (Folliculine Cristallise) A' Hautes Doses Chez Une Femme Atteinte De Prurit Vulvaire Sine Materia, *Sang* 15: 528, 1943.
8. Herbst, W. P.: Thoughts and Observations on the Relation of Altered Hormonal Balance to Prostatic Malignancy, *Urol. & Cutan. Rev.* 46: 691, 1942.
9. Elias, H., and Schwimmer, D.: Hepatotoxic Action of Diethylstilbestrol, With Report of Case, *Am. J. M. Sc.* 209: 602, 1945.
10. Seligman, B.: Hepatitis Following the Use of Diethylstilbestrol, *Urol. & Cutan. Rev.* 48: 319, 1944.
11. Arnold, O.: Ueber die Wirkung des synthetischen Brunststoffes Diäthylstilbüstrol auf das Knochenmark und Blut des Hundes, *Klin. Wehnschr.* 18: 891, 1939.

12. Tyslowitz, R., and Dingemanse, E.: Effect of Large Doses of Estrogens on the Blood Picture of Dogs, *Endocrinology* 29: 817, 1941.
13. Crafts, R. C.: Effects of Endocrine on Formed Elements of Blood; Effect of Estrogens in Dog and Monkey, *Endocrinology* 29: 606, 1941.
14. Castrodale, D., Bierbaum, O., Helwig, E. B., and MacBryde, C. M.: Comparative Studies of Effects of Estradiol and Stilbestrol Upon Blood, Liver, and Bone Marrow, *Endocrinology* 29: 363, 1941.
15. von Haam, E., Hammel, M. A., Rardin, T. E., and Schoene, R. H.: Experimental Studies on Activity and Toxicity of Stilbestrol, *Endocrinology* 28: 263, 1941.
16. Page, R. C., Russell, H. K., and Schwabe, E. L.: Chronic Toxicity Studies of Diethylstilbestrol; Subcutaneous Implantation of Pellets in Rats, *Endocrinology* 29: 230, 1941.
17. Jacobson, L. O.: Effect of Estrogens (Estradiol Benzoate) on Peripheral Blood and Bone Marrow of Mice, *Endocrinology* 34: 240, 1944.
18. Tyslowitz, R., and Hartman, C. G.: Influence of Large Doses of Estrogens on Blood Picture of Rhesus Monkeys (*Macaca Mulatta*), *Endocrinology* 29: 349, 1941.
19. Roberts, H. K., Helwig, E. B., Elman, R., and MacBryde, C. M.: Serial Biopsy Studies of Effects of Estrogens on Liver; Chemical and Morphologic Responses to Diethylstilbestrol and Estradiol, *J. Lab. & Clin. Med.* 29: 1062, 1944.
20. American Medical Association Council on Pharmacy and Chemistry: Report on Benzestrol, *J. A. M. A.* 126: 1085, 1944.
21. Minot, G. R.: Purpura Hemorrhagica With Lymphocytosis: an Acute Type and an Intermittent Menstrual Type, *Am. J. M. Sc.* 192: 445, 1936.
22. Nagy, G.: Ist es Berechtigt im Rahmen der hämorrhagischen Diathesen eine "Purpura dysovarica" als selbständiges Krankheitsbild anzunehmen? Kastrationsversuche mit Ovarialbestrahlungen beim chronischen Werlhof, *Ztschr. f. klin. Med.* 102: 284, 1926.
23. David, W.: Ueber "Purpura" Erkrankungen bei Frauen, *Med. Klin.* 22: 1755, 1926.
24. Leschke, E., and Wittkower, E.: Der Werlhofsche Blutfleckenkrankheit (thrombopenische Purpura). Ein Beitrag zur Pathologie der Blutplättchen und Capillaren und zur Pathogenese der hämorrhagischen Diathese, *Ztschr. f. klin. Med.* 102: 649, 1926.
25. Ehrenberg, C.: Ueber einen Fall von essentieller Thrombopenie und seine Behandlung durch Milzexstirpation, *Monatschr. f. Geburtsh. u. Gynäk.* 51: 99, 1920.
26. Goldburgh, H. L., and Gouley, B. A.: Postpubertal Menorrhagia and Its Possible Relations to Thrombocytopenic Purpura Hemorrhagica, *Am. J. M. Sc.* 200: 499, 1940.
27. Rushmore, S.: Purpura Complicating Pregnancy, *Am. J. Obst. & Gynec.* 10: 553, 1925.
28. Evans, H., and Perry, K. M. A.: Thrombocytopenic Purpura, *Lancet* 2: 410, 1943.
29. Ellman, P., and Weber, F. P.: Recurrent Menstrual Purpura and Vicarious Menstruation, *Brit. J. Dermat.* 47: 197, 1935.
30. Smith, C. E.: Menstrual Purpura, *New Orleans M. & S. J.* 90: 214, 1937.
31. Genell, S.: A Study of Variations in the Number of Blood-Platelets During the Menstrual Cycle, *J. Obst. & Gynaec. Brit. Emp.* 43: 1124, 1936.
32. Pohle, F. J.: Blood Platelet Count in Relation to Menstrual Cycle in Normal Women, *Am. J. M. Sc.* 197: 40, 1939.
33. Dameshek, W.: Method for Simultaneous Enumeration of Blood Platelets and Reticulocytes, With Consideration of Normal Blood Platelet Count in Men and in Women, *Arch. Int. Med.* 50: 579, 1932.
34. Zondek, B., and Bromberg, Y. M.: Endocrine Allergy. I. Allergic Sensitivity to Endogenous Hormones, *J. Allergy* 16: 1, 1945.
35. Zondek, B.: Endocrine Allergy, *J. Allergy*. In press.

## ESSENTIAL THROMBOCYTOPENIC PURPURA

### AUTOPSY FINDINGS IN THIRTY-SIX CASES

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THE files of the pathology department of the University of Minnesota contain the records of over 51,000 autopsies. Most of the patients with purpura listed are secondary types following such diseases as leukemia, meningococcic sepsis, bacterial endocarditis, scarlet fever, measles. Some of them are examples of drug idiosyncrasies. After eliminating these cases, there remained thirty-six that belonged to the essential or idiopathic group. Wiseman, Doan, and Wilson<sup>1</sup> and Wintrobe<sup>2</sup> have given rigid criteria for the definition of essential thrombocytopenic purpura from the clinical and anatomical viewpoint.

The average age of these thirty-six patients was 30.5 years. Women predominated, twenty-four to twelve men. This confirms the findings in the literature that essential thrombocytopenic purpura is primarily a disease of young people and that the women show a marked preponderance. It is possible that the physiologic decrease in the number of platelets during menstruation may be a predisposing factor for the development of the signs of purpura in women when associated with other causative factors that decrease the platelet level. Hormones may be another factor. The average platelet count in these cases was 55,000 per cubic millimeter. The bleeding times varied from ten minutes to four hours, the coagulation time from three to eight minutes.

The gross findings at autopsy, apart from the hemorrhages that have resulted in the death of the patient, are meager. The body may have the anemic appearance that follows profuse or prolonged hemorrhage. The degree of anemia may be very severe. The hemorrhages may be so widespread that it is difficult to consider any part of the body as the principal site of the bleeding. There were twelve such cases in the total number of thirty-six. In twelve of these death was due to intracranial hemorrhages. Unconsciousness and death may occur within a short time after the onset of acute purpura. Most of the hemorrhages were located beneath the dura mater and were diffusely scattered over the surfaces of the brain. At times it was difficult to decide whether the hemorrhage was extrinsic or intrinsic to the arachnoid membrane. The fact that some of the hemorrhages had the appearance of a subdural hematoma with a tendency towards organization of the clot to the inner surface of the dura would tend to show that most of the hemorrhages are subdural rather than subarachnoid in origin. Occasionally intracerebral hemorrhages may occur with or without an associated subdural hemorrhage. The intracerebral and intracerebellar hemorrhage may vary from petechiae to massive hemorrhages. Once the diagnosis of essential thrombocytopenic purpura is established the

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risk of death from fatal hemorrhage is probably higher than the operative mortality of a splenectomy in the hands of capable surgeons.

In two cases bleeding from the gastrointestinal tract predominated. The hemorrhages varied from massive ecchymosis to petechiae involving the mucosa, walls, and serosa of the stomach, intestine, and colon. In one case the principal bleeding was from the urinary tract. The bleeding was largely from the mucosa of the renal pelvis and bladder. Large perirenal hematomas were not uncommon in other cases. Nine patients continued to suffer from widespread hemorrhages following splenectomies. In one patient, who died after a splenectomy, an accessory spleen that weighed 12 grams<sup>3</sup> was found at autopsy.

The earlier investigators centered their interest largely upon the spleen. The normal adult spleen weighs approximately 150 grams. The average weight of twenty spleens in patients over 20 years of age in this series was 232 grams. The smallest weighed 103 grams and the largest 365 grams. It is seen that the spleen is only slightly enlarged and is not palpable. Pemberton<sup>4</sup> in a series of fifty-seven splenectomies for thrombocytopenic purpura found the average weight of the spleen to be 201.25 grams. However, he included in this series a spleen which weighed 700 grams in a 10-year-old child.

The histologic findings in the spleen are negligible. The majority of spleens had a fairly normal histologic structure. The Malpighian corpuscles are prominent as observed commonly in young people. The sinuses of the pulp are frequently filled and distended with small groups of red blood cells. Small collections of neutrophils are fairly common. However, this is not sufficient to speak of a true splenitis. Slight fibrosis of the pulp was observed in one case. Nickerson and Sunderland<sup>5</sup> in five patients with essential thrombocytopenic purpura attached importance to the finding of megakaryocytes in the sinuses of the pulp. Megakaryocytes are occasionally seen in the sinuses of the pulp. The bone marrow in these patients usually contained large numbers of similar cells. Younger forms of megakaryocytes are difficult to distinguish from large reticulum cells of the splenic sinuses in the usual hematoxylin and eosin preparations. The lack of other signs of myeloid metaplasia in the spleen would indicate that these megakaryocytes are embolic from the bone marrow. Kaznelson<sup>6</sup> reported finding phagocytosis of the platelets by the reticulo-endothelial cells of the splenic sinuses. Others have failed to find this phenomenon. It is extremely difficult to study phagocytosis of platelets in paraffin sections. The study of smears from the splenic vein at time of operation and imprints of the spleen before fixation would be a distinct aid in this direction. One can conclude that the spleen shows no gross or microscopic changes which are characteristic of thrombocytopenic purpura.

In recent years, the value of bone marrow studies in essential thrombocytopenic purpura has been widely recognized. Post-mortem autolysis and embalming usually restrict bone marrow studies at autopsy to paraffin sections of fixed and decalcified material. Hence, one is at a disadvantage in studying minute cellular detail as compared to fresh material in the living patient stained with some modification of the Romanowsky stain. Unfortunately in these thirty-six patients, the bone marrow was examined in only ten. Sections

were available for study in eight of the ten. In the patients not available for study, the bone marrow was described as negative for leukemia and normal megakaryocytes. In the remaining eight, three died following splenectomy. These three patients showed a marked reduction in the number of megakaryocytes in the marrow. We are rapidly beginning to realize that in any patient with thrombocytopenic purpura, where the marrow shows a marked decrease in number of megakaryocytes, the patient is likely to receive little benefit from a splenectomy. This is well illustrated by the following case in this series.

A woman, 22 years of age, gave a history of bleeding from gums, vagina, and skin purpura beginning in February of 1942. Red blood count on admission to the hospital was 1,260,000 cells per cubic millimeter; hemoglobin, 33 per cent (Sahli); leukocyte count, 7,700 cells per cubic millimeter with 69 per cent neutrophils; platelet count, 55,000 per cubic millimeter; bleeding time, over 16 minutes; and coagulation time, 4 minutes. Three bone marrow aspirations showed an absence of megakaryocytes and no evidence of leukemia. The patient was given six blood transfusions. On Feb. 28, 1942, a splenectomy was done as a possible lifesaving procedure. The platelet count rose but declined on the second post-operative day with a return of purpuric bleeding. The patient died on the fifth post-operative day from generalized purpura. The bone marrow at autopsy showed an absence of megakaryocytes.

The bone marrow in the five patients not operated upon showed a marked increase in the number of megakaryocytes with the presence of many immature megakaryocytes. Every high power microscopic field had from one to several large megakaryocytes. The degree of platelet production on the part of megakaryocytes could not be determined in the fixed tissue. These marrows otherwise appeared relatively normal except for a compensatory normoblastic erythroid hyperplasia and myeloid hyperplasia compatible with a chronic bleeding state. Eosinophilia was not noted. The question arises as to whether one should restrict the definition of essential thrombocytopenic purpura to only those cases that show a marked increase in number of megakaryocytes in the bone marrow together with the presence of immature megakaryocytes. However, some investigators, as Rosenthal,<sup>7</sup> refer to one type of idiopathic thrombocytopenic purpura where megakaryocytes are reduced in number. One would expect poor results from splenectomies in these cases.

We are still ignorant as to the nature of the capillary defect and relationship of the spleen, platelets, and capillaries in this disease. The large numbers of megakaryocytes in the bone marrow would tend to show, as emphasized by Limarzi and Schleicher<sup>8</sup> and Dameshek and Miller,<sup>9</sup> that the lack of platelets is the result of a splenic depressing factor that interferes with the proper maturation of the megakaryocytes in the bone marrow.

#### CONCLUSIONS

1. The post-mortem findings of thirty-six patients with essential thrombocytopenic purpura are recorded.
2. The average weight of the spleen in twenty adults was 232 grams. There were no characteristic histologic findings.
3. Apart from the widespread hemorrhages, the presence of increased number of megakaryocytes in the bone marrow is the most constant post-mortem finding in essential thrombocytopenic purpura.

## REFERENCES

1. Wiseman, B. K., Doan, C. A., and Wilson, S. J.: The Present Status of Thrombocytopenic Purpura, With Special Reference to Diagnosis and Treatment, *J. A. M. A.* 115: 8-13, 1940.
2. Wintrobe, M. M.: *Clinical Hematology*, Philadelphia, 1946, Lea & Febiger, 611 pp.
3. Watson, C. J., and Moir W. W., Jr.: Recurrence of Thrombocytopenic Purpura After Splenectomy in a Case With an Accessory Spleen, *Internat. Clin.* 4: 221-230, 1941.
4. Pemberton, J. deJ.: The Diagnosis and Treatment of Purpura Hemorrhagica, *Am. J. Surg.* 24: 793-806, 1934.
5. Nickerson, D. A., and Sunderland, D. A.: The Histopathology of Idiopathic Thrombocytopenic Purpura Hemorrhagica, *Am. J. Path.* 13: 463-490, 1937.
6. Kaznelson, P.: Verschwinden der hamorrhagischen Diathese bei einem Falle von essentieller Thrombopenie (Frank) nach Milzextirpation. Splenogene thrombolytische Purpurea, *Wien. klin. Wchnschr.* 29: 1451-1454, 1916.
7. Rosenthal, N.: The Course and Treatment of Thrombocytopenic Purpura, *J. A. M. A.* 112: 101-106, 1939.
8. Limarzi, L. R., and Schleicher, E. M.: The Reaction of Peripheral Blood and Bone Marrow in Chronic Hemorrhage and in Essential Thrombocytopenic Purpura, *J. A. M. A.* 114: 12-18, 1940.
9. Dameshek, W., and Miller, E. B.: The Megakaryocytes in Idiopathic Thrombocytopenic Purpura, a Form of Hypersplenism, *Blood* 1: 27-51, 1946.



# EXPERIENCE WITH PTEROYLGLUTAMIC (SYNTHETIC FOLIC) ACID IN THE TREATMENT OF PERNICIOUS ANEMIA

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THE synthesis of a compound known to have a specific stimulating effect on hematopoiesis in man and in certain laboratory animals was announced in August, 1945, by a group of sixteen investigators.<sup>1</sup> This substance, synthetic folic acid, was found to be identical with the *Lactobacillus casei* factor previously derived in pure form from liver.<sup>2</sup> Extracts containing a substance or substances essential to the growth of *Lactobacillus casei* (and *Streptococcus lactis* R) have been obtained from sources<sup>3-6</sup> other than liver, namely, kidney, yeast, and the green leaves of many plants, notably spinach. As yet it is not known, however, whether the active principle in these extracts is identical with the synthetic compound or with the factor derived from liver.<sup>6</sup> Spies and associates<sup>7</sup> considered synthetic folic acid to be a part of the vitamin B complex. The formula and the method of synthesis of the hepatic *L. casei* factor were published in May, 1946; "pteroylglutamic acid" was suggested as a name for this compound.<sup>8</sup>

In recent months reports have been published on the therapeutic effectiveness of pteroylglutamic (synthetic folic) acid in certain macrocytic anemias. The rationale of use of this substance in the treatment of anemias of the macrocytic type was based on the experimental work of Wills and associates<sup>9, 10</sup> in England and of Day and co-workers<sup>11-14</sup> in this country. These workers produced a dietary deficiency in monkeys characterized by macrocytic anemia, leukopenia, diarrhea, and oral lesions (necrosis and ulceration of the gums), a syndrome not unlike that of sprue in man. Day and co-workers postulated that the deficiency was due to an unknown substance for which the term "vitamin M" was proposed. Subsequently, the same group of investigators demonstrated that deficiency of vitamin M in monkeys could be treated successfully with a highly purified *L. casei* factor.<sup>14, 15</sup>

The similarities of the sprue syndrome in man to the manifestations of deficiency of vitamin M in monkeys led two groups of investigators, Spies and associates and Darby and co-workers, to study the effect of the synthetic compound in patients with sprue.<sup>7, 16-22</sup> Clinical improvement characterized by disappearance of the glossitis, regeneration of lingual papillae, subsidence of diarrhea, and gain in weight was noted. Concomitantly, a marked hematologic response manifested by reticulocytosis and a rise in values for hemoglobin and erythrocytes was observed.

Studies on the therapeutic value of pteroylglutamic acid in other types of macrocytic anemia—addisonian pernicious anemia, pernicious anemia of pregnancy, nutritional macrocytic anemia, and macrocytic anemia of infancy—now have been reported also.<sup>7, 19, 23-25</sup> Vilter and associates<sup>16</sup> and Moore and co-

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The pteroylglutamic (synthetic folic) acid employed in this study was furnished by Dr. Stanton M. Hardy, of the Lederle Laboratories, Inc., Pearl River, N. Y.

\*Reference to vitamin B<sub>12</sub> and allied substances has been omitted, since evidence indicates that, although related, they probably are not identical with pteroylglutamic acid. The physiologic action of vitamin B<sub>12</sub> on hematopoiesis, however, appears to be similar to that of pteroylglutamic acid.

workers<sup>23</sup> reported simultaneously in December, 1945, on the effect of pteroylglutamic acid in addisonian pernicious anemia in relapse. To the results observed in the seven cases reported by these investigators, Doan and associates<sup>24, 26</sup> have added their observations in four cases, Kaufmann and Schwager<sup>27</sup> in two cases, Amill and Wright<sup>28</sup> in six cases, and Goldsmith<sup>29</sup> in four cases, making a total of twenty-three cases of pernicious anemia in relapse on which published data concerning the effects of pteroylglutamic acid therapy are available. In these twenty-three cases the administration of pteroylglutamic acid, orally or parenterally, resulted in a reticulocyte response, an increase in values for hemoglobin and erythrocytes, and clinical improvement. Although studies on the effects of this form of therapy on the bone marrow of patients who had pernicious anemia in relapse have been made in several of the reported cases, detailed studies are available in only two instances.<sup>24, 26</sup> In these cases the megaloblastic marrow of pernicious anemia in relapse was converted to a marrow which approaches normal from the tenth to the fourteenth day of treatment.

It is the purpose of this paper to record our observations on the effects of pteroylglutamic acid therapy in fourteen patients with classical addisonian pernicious anemia in relapse. We wish to emphasize particularly the changes observed in the bone marrow after the administration of this substance and the failure of the synthetic compound to relieve or prevent the development of gastrointestinal and neurologic manifestations of the disease.

#### MATERIAL AND METHODS

In nine of the fourteen cases included in this study the condition in question was seen for the first time, the diagnosis having been made just prior to treatment with pteroylglutamic acid. In the remaining five cases (Patients 1, 2, 3, 7, and 14) a sensitivity to liver extracts previously administered parenterally had developed in two (Patients 7 and 14), and the patients were in severe relapse at the time of their admission to the Mayo Clinic. Two others (Patients 2 and 3) in this group of five had been informed one year and three years previously that they had pernicious anemia, but they had neglected liver therapy over periods of many months. The fifth (Patient 1) had received a single intramuscular injection of liver extract one week before admission because his physician told him that he was anemic, although the diagnosis of pernicious anemia apparently had not been established. Eight of the fourteen patients were women and six were men. Their ages ranged from 48 to 72 years. Six of the fourteen patients were hospitalized during the first four to six weeks of treatment and were given a routine hospital diet. The remaining eight patients were seen daily or every second day in the clinic and were maintained on diets of an average protein content (70 Gm.).

During the first two weeks of treatment erythrocyte and reticulocyte counts were made daily; subsequently, such counts were made every second or third day. Reticulocyte percentages were obtained by counting 1,000 cells on dry films stained with brilliant cresyl blue and counterstained with Wright's stain (Grübler). The Sheard-Sanford photometer was used for the determination of values for hemoglobin, and a Haden erythrocytometer was employed for the determination of average erythrocyte diameters. Sternal puncture was performed in twelve of the fourteen cases prior to the institution of therapy and was repeated at intervals in each case, so that in the aggregate at least one puncture was performed for each day from the second to the tenth day of treatment. Both the oral route and the parenteral route of administration of pteroylglutamic acid were employed.

#### RESULTS

*Hematologic Response.*—The quantity of pteroylglutamic acid administered, the route of administration, and the hematologic response in respect to

each of the fourteen patients is shown in Table 1. Peaks of the increase in reticulocytes occurred from the fifth to the fourteenth day after the institution of therapy, and the increases varied from 1.6 to 17.6 per cent. These values, in general, are somewhat lower than those reported by other observers and are less than the reticulocyte peaks observed after liver therapy among patients who had pernicious anemia in similar states of relapse.

Although treatment with pteroylglutamic acid resulted in an increase in values for erythrocytes and hemoglobin in all cases, the rapidity of the increase was variable. In some cases the response appeared to be as rapid as that usually observed with liver therapy (Fig. 1), but in other cases it was decidedly delayed (Fig. 2). In three patients the values for erythrocytes did not increase above 3,500,000 cells per cubic millimeter of blood, despite daily doses of 15 to 20 mg. over periods as long as eleven weeks (Patients 2, 9, and 13). In these three patients normal blood values subsequently were induced with liver therapy. Recession in the degree of macrocytosis occurred in all cases, but a return to essentially normal cell diameters was observed in only three instances (Patients 4, 11, and 14).

Leukocyte counts of less than 5,000 cells per cubic millimeter were observed in six of the fourteen patients prior to treatment with pteroylglutamic acid. Treatment resulted in an increase in the number of leukocytes to normal in two cases (Patients 1 and 7), an increase with persistence of a subnormal value in two (Patients 8 and 14), and a decrease in two (Patients 2 and 12). Platelet determinations made prior to treatment with pteroylglutamic acid revealed thrombocytopenia in four of seven patients. After treatment, an increase in platelet values occurred in all four patients.

In the twelve patients for whom sternal puncture was performed megaloblastic regeneration in the bone marrow was found prior to treatment with folic acid. Treatment resulted in a rapid conversion to a normoblastic marrow, the conversion being essentially complete in seven to ten days. The changes observed are illustrated in material aspirated serially in a typical instance (Figs. 3 to 5).

*Clinical Observations.*—Although evaluation of subjective symptoms is more difficult than analysis of objective data, the impression was gained that in pernicious anemia in relapse treatment with pteroylglutamic acid resulted in less pronounced and less rapid subjective improvement than that commonly observed after the institution of liver therapy. With the exception of two cases in which the hematologic response to pteroylglutamic acid therapy was rapid, weakness persisted longer and the restoration of a sense of well-being, which often occurs with startling rapidity after the administration of liver extract, either was significantly delayed or did not occur.

The results of treatment with pteroylglutamic acid over periods of many months on the glossitis and neural manifestations of pernicious anemia are summarized in Table II. Of seven patients who had glossitis, six improved under treatment; however, the improvement of four of these was slower than that observed with liver therapy, since one to three and a half months of therapy with acid were required for restoration of a normal tongue. Three of the six patients experienced recurrence of glossitis at a later date; in two of them (Patients 2 and 7) satisfactory hematologic remission was not induced, but in

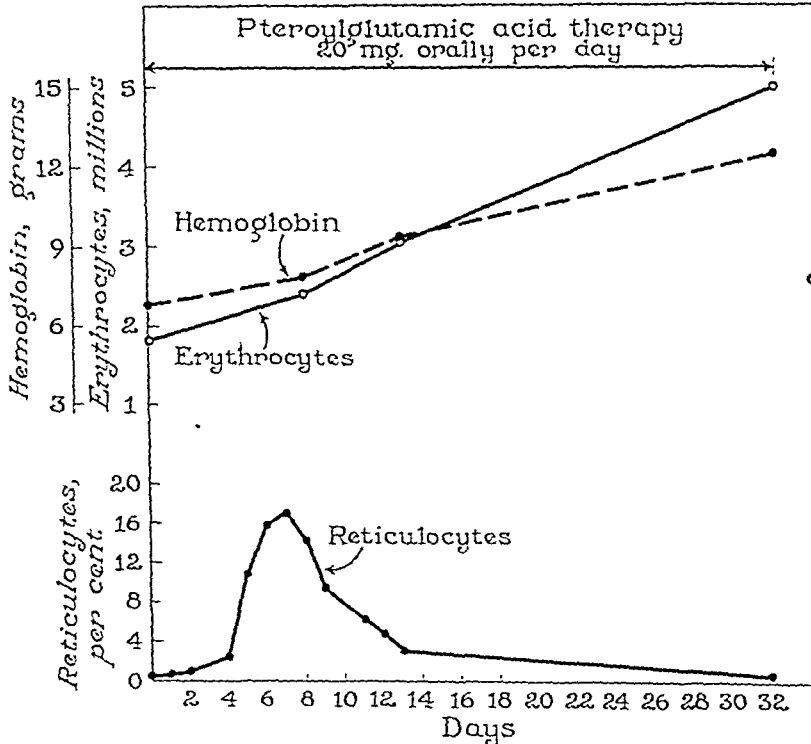


Fig. 1.—A satisfactory erythrocytic response (Patient 14) to pteroylglutamic acid therapy in pernicious anemia in relapse. The increase in values for erythrocytes and hemoglobin was rapid, and the reticulocyte response appeared to be either maximal or very nearly maximal.

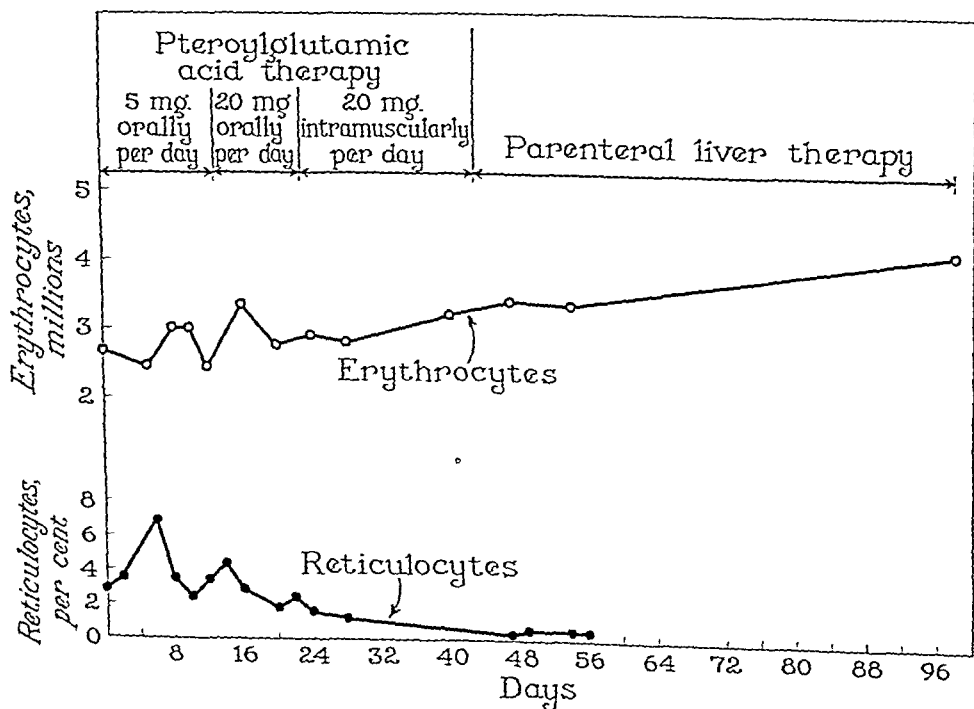


Fig. 2.—An unsatisfactory erythrocytic response (Patient 13) to pteroylglutamic acid therapy in pernicious anemia in relapse. Only a slight increase in the number of erythrocytes occurred over the first four days. The initial submaximal reticulocyte response may have been caused by the small dose administered during the first eleven days of treatment. Larger doses both orally and parenterally did not result in a significant regeneration of erythrocytes. Therapy with liver extract administered brought about restitution of normal blood values.

TABLE I. DOSAGE, ROUTE OF ADMINISTRATION, AND HEMATOLOGIC RESPONSE TO PTEROYLGLUTAMIC ACID THERAPY IN PERNICIOUS ANEMIA

PATIENT	AGE	SEX	PTEROYLGLUTAMIC ACID THERAPY			HEMOGLOBIN (GRAMS)		ERYTHROCYTES (MILLIONS)				RETICULOCYTES			ERYTHROCYTEMEETER READINGS (MICRONS)		LEUKOCYTES (THOUSANDS)		PLATELETS (THOUSANDS)	
			ADMINISTRATION (ROUTE)	DAILY DOSE (MG.)	DURATION OF TREATMENT (DAYS)	INITIAL	FINAL	INITIAL	FOURTEENTH DAY	THIRTIETH DAY	FINAL	FIRST DAY OF RISE	DAY OF PEAK	PERCENT AT PEAK	INITIAL	FINAL	INITIAL	FINAL	INITIAL	FINAL
1	65	M	Oral	100	1 to 37	10.1	13.1	2.44	3.32	3.96	4.19	8	11	6.4	8.3	7.8	4.8	6.6	202	
2	72	M	Oral	100	1 to 14	6.2	10.3	1.61	2.36	3.44	3.43	3	5	10.2	8.6	7.5	4.4	2.7		119
3	66	M	Oral	50	1 to 14					(13) <sup>*</sup>										
			Oral	15	15 to 71	12.0	11.9	3.21	3.51	3.88	3.94	5	8	6.3	8.0	7.7	6.8	8.9	176	147
4	64	F	Oral	10	72 to 147					(32)	4.27	3	6	8.9	8.0	7.1	8.1	9.3	185	119
			Oral	5	34 to 99	10.0	13.1	2.72	3.71											
			Oral	10	100 to 165															
			Oral	20	166 to 213															
5	66	M	Oral	25	1 to 14					(26)										106
			Oral	15	15 to 27	9.8	10.8	2.78	3.22	3.49	3.97	5	11	3.7	8.4	7.5	7.7	8.6		
			Oral	20	28 to 96															
			Oral	10	97 to 218															
6	52	F	I.M.	20	1 to 21					(21)	4.22	6	12	4.5	8.3	7.8	6.5	11.5	58	163
			Oral	10	22 to 55	5.3	13.8	1.28	3.25	4.15										
			Oral	5	56 to 261															

\*Numbers in parentheses denote day on which determination was made, if different from thirtieth day.

7	68	P	I.M. I.M. Oral	10 20 15	1 to 20 21 to 30 31 to 160	4.0	11.2	1.15	2.43	(28) 3.20	3.21	3	5	17.6	8.3	7.7	3.4	6.5	67	130
8	67	P	I.M. I.M. Oral Oral Oral Oral	20 10 10 25 15	1 to 14 15 to 26 27 to 41 42 to 43 47 to 55	3.7	11.7	1.00	2.76	3.47	4.52	4	9	13.8	8.1	7.7	2.0	4.7	80	116
9	66	P	I.M.	20	1 to 24	10.5	11.6	3.05	3.23	3.38		7	8	4.5	8.3	8.0	4.0			
10	65	M	I.M. Oral	20 5	1 to 42 1 to 10	11.2	11.9	2.57	3.24	3.99		3	7	4.5	8.3		7.3			
11	50	P	Oral Oral	20 10	11 to 68 69 to 167	8.7	13.8	2.40	3.13	3.97	4.68		7	14.4	8.0	7.2	6.1	6.9		
12	60	P	I.M. I.M. Oral Oral Oral	10 20 20 15 30	1 to 10 11 to 25 26 to 70 71 to 136 137 to 193	13.3	12.9	3.69	3.84	3.54	4.20	4	4	1.6		7.0	7.2	4.8		
13	48	P	Oral Oral I.M.	5 20 20	1 to 11 12 to 23 24 to 44	8.9		2.67	2.65	(28) 2.80	3.21	3	7	6.9	8.0	7.8	7.2	4.9	171	119
14	55	M	Oral	20	1 to 32	6.5	14.0	1.44	3.06	(32) 5.01	4.82	5	8	16.7	8.0	7.2	3.9	4.5	55	166

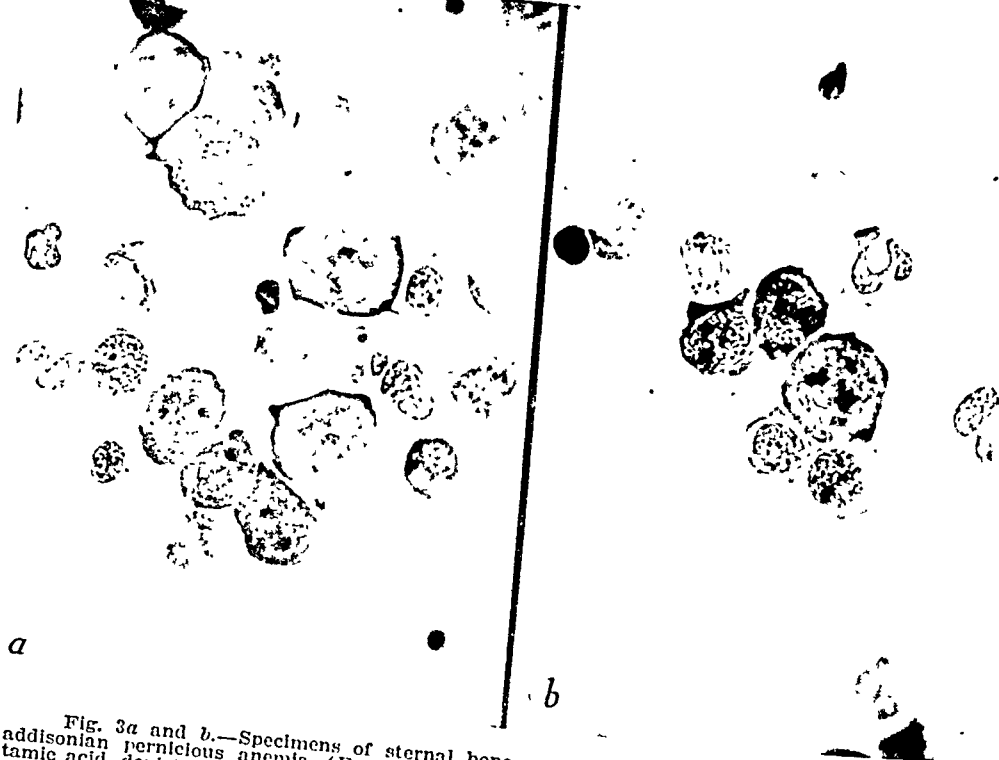


Fig. 3a and b.—Specimens of sternal bone marrow aspirated serially in a patient with Addisonian pernicious anemia (Patient 2), during relapse before treatment with pteroylglutamic acid, depicting a predominance of promegaloblasts and basophilic megaloblasts ( $\times 650$ ).

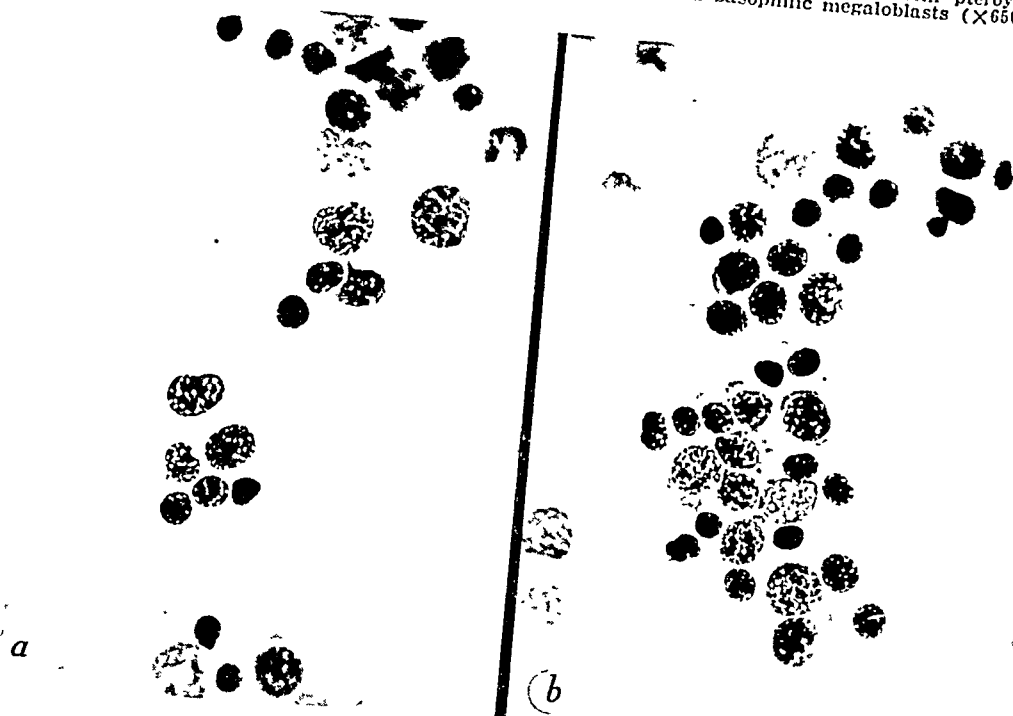


Fig. 4a and b.—a, Same case as concerned in Fig. 3. Specimen of sternal marrow three days after the institution of pteroylglutamic acid therapy, showing late megaloblastic maturation, manifested by a predominance of orthochromatic megaloblasts ( $\times 650$ ); b, normoblastic regeneration in same specimen as shown in Fig. 4a, as evidenced by basophilic, polychromatic, and orthochromatic normoblasts ( $\times 650$ ).

the third (Patient 8) the blood picture was essentially normal at the time of recurrence of the glossitis except for persistence of a slight degree of macrocytosis. The one (Patient 9) who obtained no abatement of the glossitis was treated with pteroylglutamic acid for only twenty-four days. Although therapy apparently failed in this instance, the period of observation was too short to permit the formation of conclusions.



Fig. 5a and b.—a, Same case as concerned in Figs. 3 and 4, showing specimen of sternal bone marrow eight days after institution of pteroylglutamic acid therapy, depicting active normoblastic regeneration; a few scattered megaloblasts could still be found at this stage ( $\times 650$ ); b, specimen of sternal marrow thirty-four days after the institution of pteroylglutamic acid therapy, showing resumption of active myelopoiesis as well as less intensive normoblastic regeneration ( $\times 650$ ).

Of the ten patients who had paresthesia in the extremities, eight improved, but the improvement of four patients was temporary. It is of interest that paresthesia recurred within two to four months after the institution of therapy, at a time when three of the four patients no longer were anemic (Patients 4, 5, and 6). Of later interest is the observation that in two patients



TABLE II. EFFECT OF ADMINISTRATION OF PTEROYLGLUTAMIC ACID ON GLOSSITIS AND NEURAL MANIFESTATIONS IN PERNICIOUS ANEMIA\*

SIGN OR SYMPTOM	INSTANCES OBSERVED				
	BEFORE TREATMENT	AFTER TREATMENT			FIRST APPEAR- ANCE DURING TREATMENT
		IMPROVED, NO RECURRENCE	IMPROVED TEMPO- RARILY, RECURRENCE	UNIMPROVED OR WORSE	
Glossitis	7	3	3	1†	0
Paresthesia	10	4	4	2	2
Subacute combined de- generation of the spinal cord	2	0	0	2†	3
Total	19	7	7	5	

\*Based on the study of fourteen patients.

†Short period of observation.

who had not had paresthesia prior to treatment, this symptom developed five months after treatment had been started (Patients 3 and 6).

The condition of two patients who had peripheral neuropathy and early signs of subacute combined degeneration of the spinal cord (Patients 1 and 10) did not improve during five or six weeks of treatment. The size of the dose of pteroylglutamic acid administered to one of these patients was five times greater (100 mg. daily) than that administered to the other, but during the short period the patient receiving the larger dose was under observation before treatment with liver extract was begun, the neural manifestations of the disease were not altered. Of particular significance was the development of early but definite signs of subacute combined degeneration of the spinal cord in three patients who had not had involvement of the central nervous system prior to treatment with pteroylglutamic acid (Patients 3, 7, and 8). Two, however, had paresthesia in the extremities which abated temporarily after treatment and then recurred two to four months later. Signs of involvement of the spinal cord in these cases were loss of sense of vibration and sense of position in the lower limbs, slight incoordination, presence of the Romberg sign, slight but definite ataxia, and, in one patient, the bilateral presence of the Babinski great toe sign. The neural manifestations developed within two to five months after treatment with pteroylglutamic acid had begun. In one case (Patient 8) they developed at a time when the number of erythrocytes was normal, although a slight degree of macrocytosis persisted. In another (Patient 3), in which the patient who had not had symptoms or signs of involvement of the nervous system prior to treatment with pteroylglutamic acid, a satisfactory hematologic remission was not induced, as manifested by persistence of macrocytosis and a value for erythrocytes that did not increase above 4,000,000 cells per cubic millimeter of blood, except on one occasion. One case (Patient 7) is of special interest because the erythrocyte count increased from 1,150,500 to 4,000,000 cells per cubic millimeter over a period of four months and then decreased to 3,210,000 cells per cubic millimeter in the next five weeks (Fig. 6). During the period of decrease in number of erythrocytes and with the patient receiving a daily maintenance dose of 15 mg. of pteroylglutamic acid by mouth, glossitis and paresthesia in the extremities recurred and signs of subacute combined degeneration of the spinal cord developed. Inquiry into the diets of three patients revealed essentially normal diets in two (Patients 3 and 8), but the

diet in one (Patient 7) was low in protein. In the latter instance the patient estimated that she had not received in excess of a half pound of animal protein weekly for a period of two months prior to the development of glossitis and the symptoms of involvement of the nervous system. She attributed the low intake of protein to the meat shortage prevailing during the summer of 1946.

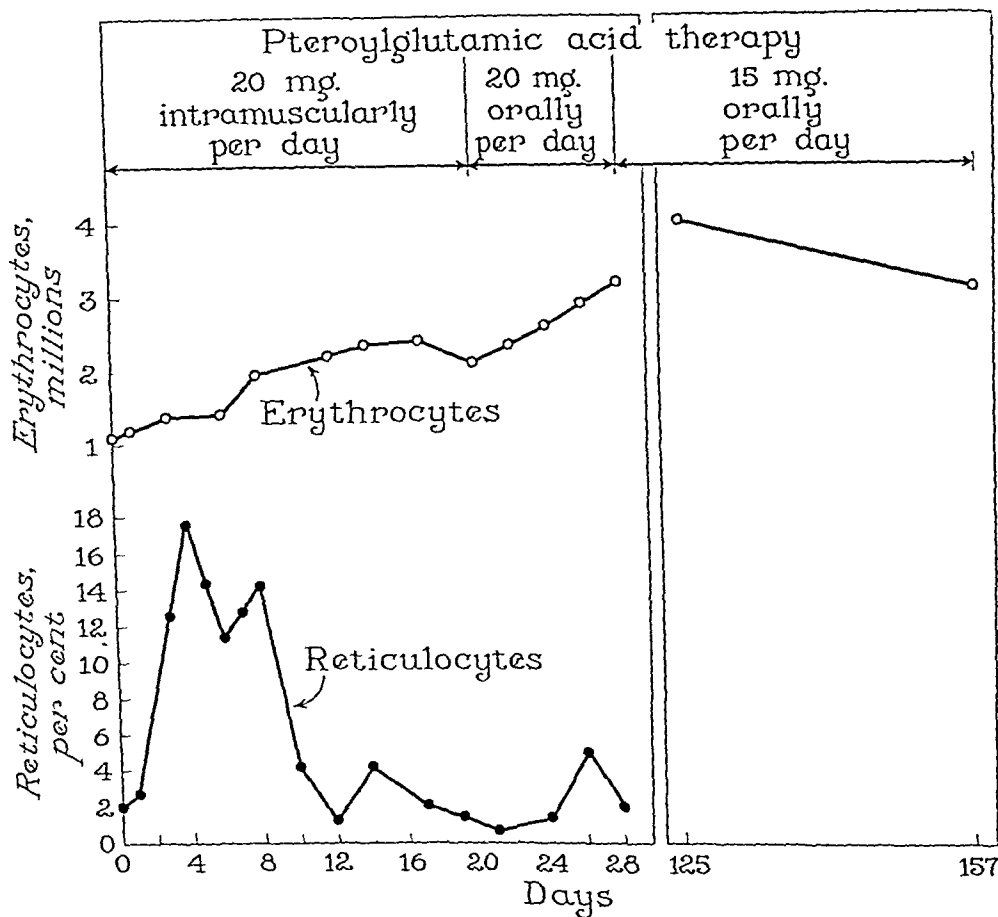


Fig. 6.—Diminution of the number of erythrocytes per cubic millimeter of blood in a patient with pernicious anemia receiving orally a daily dose of 15 mg. of pteroylglutamic acid, after an apparently satisfactory initial response to such therapy. In this case (Patient 7) relapse was due to cessation of liver therapy, many months prior to the patient's admission, because of sensitivity to liver extracts. The presenting symptoms were glossitis and paresthesia in the lower extremities, but the signs of combined systemic disease were absent. During the first two months of treatment the glossitis and paresthesia abated but then re-occurred and became moderately severe during the last few weeks of treatment. During the period of the decrease in erythrocytes, the early signs of subacute combined degeneration of the spinal cord developed. Desensitization of the patient to liver extract administered parenterally then was carried out, and intensive liver therapy subsequently resulted in restitution or normal blood values and symptomatic improvement.

#### COMMENT

Pteroylglutamic acid appears to be an essential nutrient for man, for various laboratory animals, and for certain microorganisms. An important physiologic property is the stimulating effect of the acid on hematopoiesis, which is most pronounced in certain, but not all, types of macrocytic anemia in man characterized by megaloblastic regeneration in the bone marrow. In this type of anemia, including addisonian pernicious anemia, the administration of the

synthetic compound results in the prompt conversion of a pathologic megaloblastic form of erythropoiesis to a normal normoblastic form of erythropoiesis. Concomitant with the changes observed in the bone marrow, a hematologic response occurs, characterized by reticulocytosis and an increase in values for hemoglobin and erythrocytes. An increase in the number of leukocytes and platelets also occurs in most cases in which leukopenia and thrombocytopenia are found prior to treatment.

Although the foregoing remarks on the hematopoietic-stimulating effect of pteroylglutamic acid applied, in general, to all cases of patients with pernicious anemia in our series, considerable variation in the rate of hematologic improvement occurred. Only three of fourteen patients who had pernicious anemia in relapse treated with pteroylglutamic acid showed an erythrocyte response comparable to that which occurs after liver therapy. In the remaining eleven cases the rate of increase of erythrocytes was significantly slower than the expected response to treatment with liver extract. In four cases normal values for erythrocytes were not attained after eight to ten weeks of treatment. Subsequent intensive liver therapy, however, resulted in the restitution of normal blood values in these four cases. It is of interest to note also that in the majority of our cases the reticulocyte responses to treatment with pteroylglutamic acid were lower than the standard maximal reticulocyte responses that have been established for various erythrocyte levels after the intramuscular administration of liver extract.<sup>30-32</sup>

Clinical improvement after pteroylglutamic acid therapy, as well as the hematologic response, was found to be variable in patients who had pernicious anemia in relapse, and often it was slower than the response observed with liver therapy. In a significant proportion of cases glossitis and neurologic manifestations of the disease either failed to abate or recurred after temporary abatement. Of greater significance was the development of signs of subacute combined degeneration of the spinal cord in three of the fourteen patients treated with pteroylglutamic acid in doses of 10.0 to 15.0 mg. administered daily by mouth for periods of two to five months.\* These observations suggest that the physiologic action of pteroylglutamic acid on the alimentary tract and nervous system in pernicious anemia is not identical with that of the antipernicious anemia factor contained in liver, even though the action on the bone marrow is similar.

Although pteroylglutamic acid has antianemic properties, it is probable that this factor and the antipernicious anemia factor contained in liver are not the same. The quantity of pteroylglutamic acid contained in liver extracts is small. Clark<sup>33</sup> reported that various commercial liver extracts contain 0.25 to 0.5 micrograms of the *L. casei* factor per U.S.P. unit. Thus, a patient receiving 150 units of the antipernicious anemia factor in liver extract (10 c.c.) during the first ten days of treatment would receive only 5.5 micrograms of pteroylglutamic acid. Doses required for hemopoietic response are considerably larger. In our study the minimal amount of pteroylglutamic acid administered

\*Since this paper was written, Vilter, Vilter, and Spies, at the annual meeting of the Central Society of Clinical Pharmacology, N. Y., 1946, reported the development of combined systemic disease in four patients with pernicious anemia after five to eight months of treatment with pteroylglutamic acid. The administration of 50 to 500 mg. of the *L. casei* factor for ten to forty days did not result in subjective or objective improvement. We have learned also, in a personal communication, that L. Meyer of New York City previously had reported similar observations.

to the patient during the first ten days of treatment was 50,000 micrograms. However, hemopoietic responses have been obtained with as little as 20,000 micrograms during the first ten days of therapy.<sup>24</sup>

It is obvious that the discovery and synthesis of pteroylglutamic acid are important advances in the fields of nutrition and hematology. The observation that this substance does not prevent the development of glossitis and neural manifestations in pernicious anemia, even after the induction of hematologic remission, in no way detracts from the significance of the demonstration that deficiency of a substance of known chemical composition profoundly inhibits erythrocytic maturation, and possibly the maturation of other elements, in the bone marrow. Although present evidence indicates that pteroylglutamic acid should not be employed as a form of replacement or substitution therapy in pernicious anemia, whether in relapse or remission, data concerning the usefulness of this agent in the treatment of other types of macrocytic anemia related to pernicious anemia are accumulating rapidly. In our experience pteroylglutamic acid therapy has been found to be particularly effective in the treatment of nutritional macrocytic anemia and macrocytic anemia of infancy.

#### SUMMARY AND CONCLUSIONS

Fourteen patients who had addisonian pernicious anemia in relapse were treated with pteroylglutamic (synthetic folic) acid over periods from twenty-four days to nine months. The most striking effect of this form of therapy was observed in the bone marrow, erythropoiesis rapidly changing from a megaloblastic to a normoblastic type. However, considerable variation in the rate of erythrocytic regeneration was encountered, and in certain instances normal blood values were not obtained after several months of treatment with pteroylglutamic acid in doses generally thought to be relatively large.

Symptomatic improvement also was variable. Treatment resulted in abatement of glossitis and peripheral neuropathy in most cases, but recurrence was common among persons maintained solely on this form of therapy for a period of months. Moreover, peripheral neuropathy and subacute combined degeneration of the spinal cord developed as new manifestations in a significant proportion of cases two to five months after treatment was begun. In the light of these observations it is obvious that pteroylglutamic acid does not prevent the occurrence of degenerative disease of the peripheral nerves and spinal cord in pernicious anemia and that the use of this form of therapy as a substitute for extracts of liver or gastric mucosa subjects patients to the hazards of progression or development of the neural manifestations of the disease. On the other hand, pteroylglutamic acid has been found to be an effective therapeutic agent in certain macrocytic anemias related to pernicious anemia, a subject not considered in the present paper.

#### REFERENCES

1. Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., SubbaRow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr.: Synthesis of a Compound Identical With the L. casei Factor Isolated From Liver, *Science* 102: 227-228, 1945.
2. Stokstad, E. L. R.: Some Properties of Growth Factor for *Lactobacillus casei*, *J. Biol. Chem.* 149: 573-574, 1943.

3. Snell, E. E., and Peterson, W. H.: Growth Factors for Bacteria. X. Additional Factors Required by Certain Lactic Acid Bacteria, *J. Bact.* 39: 273-285, 1940.
4. Mitchell, H. K., Snell, E. E., and Williams, R. J.: The Concentration of "Folic Acid," *J. Am. Chem. Soc.* 63: 2284, 1941.
5. Luckey, T. D., Briggs, G. M., Jr., and Elvehjem, C. A.: The Use of *Streptococcus lactis* R for the Measurement of "Folic Acid," *J. Biol. Chem.* 152: 157-167, 1944.
6. Hutchings, B. L., Bohonos, N., and Peterson, W. H.: Growth Factors for Bacteria. XIII. Purification and Properties of an Eluate Factor Required by Certain Lactic Acid Bacteria, *J. Biol. Chem.* 141: 521-528, 1941.
7. Spies, T. D., Vilter, C. F., Koch, M. B., and Caldwell, M. H.: Observations of the Anti-anemic Properties of Synthetic Folic Acid, *South. M. J.* 38: 707-709, 1945.
8. Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., Subbarow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sikkels, J. P., and Smith, J. M., Jr.: The Structure and Synthesis of the Liver L. casei Factor, *Science* 103: 667-669, 1946.
9. Wills, L., and Bilimoria, H. S.: Studies in Pernicious Anemia of Pregnancy. V. Production of a Macrocytic Anemia in Monkeys by Deficient Feeding, *Indian J. M. Research* 20: 391-402, 1932.
10. Wills, L., and Stewart, A.: Experimental Anemia in Monkeys, With Special Reference to Macrocytic Nutritional Anemia, *Brit. J. Exper. Path.* 16: 444-453, 1935.
11. Day, P. L., Langston, W. C., and Shukers, C. F.: Leukopenia and Anemia in the Monkey Resulting From Vitamin Deficiency, *J. Nutrition* 9: 637-644, 1935.
12. Langston, W. C., Darby, W. J., Shukers, C. F., and Day, P. L.: Nutritional Cytopenia (Vitamin M Deficiency) in the Monkey, *J. Exper. Med.* 68: 923-940, 1938.
13. Day, P. L., Langston, W. C., Darby, W. J., Wahlen, J. G., and Mims, V.: Nutritional Cytopenia in Monkeys Receiving Goldberger Diet, *J. Exper. Med.* 72: 463-477, 1940.
14. Day, P. L., Mims, V., Totter, J. R., Stokstad, E. L. R., Hutchings, B. L., and Sloane, N. H.: Successful Treatment of Vitamin M Deficiency in Monkey With Highly Purified *Lactobacillus casei* Factor, *J. Biol. Chem.* 157: 423-424, 1945.
15. Day, P. L., Mims, V., and Totter, J. R.: The Relationship Between Vitamin M and the *Lactobacillus casei* Factor, *J. Biol. Chem.* 161: 45-52, 1945.
16. Vilter, C. F., Spies, T. D., and Koch, M. B.: Further Studies on Folic Acid in Treatment of Macrocytic Anemia, *South. M. J.* 38: 781-785, 1945.
17. Spies, T. D., Lopez, G. G., Menéndez, J. A., Minnich, V., and Koch, M. B.: The Effect of Folic Acid on Sprue, *South. M. J.* 39: 30-32, 1946.
18. Spies, T. D., Milanes, F., Menéndez, A., Koch, M. B., and Minnich, V.: Observations on the Treatment of Tropical Sprue With Folic Acid, *J. LAB. & CLIN. MED.* 31: 227-241, 1946.
19. Spies, T. D.: Effect of Folic Acid on Persons With Macrocytic Anemia in Relapse, *J. A. M. A.* 130: 474-477, 1946.
20. Darby, W. J., and Jones, E.: Treatment of Sprue With Synthetic L. casei Factor (Folic Acid, Vitamin M), *Proc. Soc. Exper. Biol. & Med.* 60: 259-260, 1945.
21. Darby, W. J., Jones, E., and Johnson, H. C.: The Use of Synthetic L. casei Factor in the Treatment of Sprue, *Science* 103: 108, 1946.
22. Darby, W. J., Jones, E., and Johnson, H. C.: Effect of Synthetic *Lactobacillus casei* Factor in Treatment of Sprue, *J. A. M. A.* 130: 780-786, 1946.
23. Moore, C. V., Bierbaum, O. S., Welch, A. D., and Wright, L. D.: The Activity of Synthetic *Lactobacillus casei* Factor ("Folic Acid") as an Antipernicious Anemia Substance, *J. LAB. & CLIN. MED.* 30: 1056-1069, 1945.
24. Doan, C. A., Wilson, H. E., Jr., and Wright, C. S.: Folic Acid (L. casei Factor), an Essential Panhematopoietic Factor; Experimental and Clinical Studies, *Ohio State M. J.* 42: 139-144, 1946.
25. Zuelzer, W. W., and Ogden, F. N.: Folic Acid Therapy in Macrocytic Anemias of Infancy, *Proc. Soc. Exper. Biol. & Med.* 61: 176-177, 1946.
26. Doan, C. A.: Folic Acid (Synthetic L. casei Factor), an Essential Panhematopoietic Stimulus, *Am. J. M. Sc.* 212: 257-274, 1946.
27. Kaufmann, J., and Schwager, P. G.: Oral Folic Acid Therapy in the Treatment of Pernicious (Addisonian) Anemia, *Canad. M. A. J.* 54: 539-544, 1946.
28. Amill, L. A., and Wright, M.: Synthetic Folic Acid Therapy in Pernicious Anemia, *J. A. M. A.* 131: 1201-1207, 1946.
29. Goldsmith, G. A.: Synthetic L. casei Factor (Folic Acid) in Treating Certain Anemias in Man, *Science* 104: 439, 1946.
30. Isaacs, R., and Friedman, A.: Standards for Maximum Reticulocyte Percentage After Intramuscular Liver Therapy in Pernicious Anemia, *Am. J. M. Sc.* 196: 718-719, 1938.
31. Riddle, M. C.: Pernicious Anemia; Blood Regeneration During Early Remission, *Arch. Int. Med.* 46: 417-439, 1930.
32. Doan, C. A.: Modern Medical Therapy in General Practice, Baltimore, 1940, Williams & Wilkins Company, 2903 pp.
33. Clark, G. W.: Vitamin Content of Liver Extracts for Parenteral Use; Comparison of Crude and Concentrated Preparations, *Am. J. M. Sc.* 209: 520-524, 1945.

# THE EFFECT OF SYNTHETIC LACTOBACILLUS CASEI FACTOR ON THE BLOOD CHANGES INDUCED BY GASTRECTOMY IN THE RAT

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ONE of the newest members of the group of water-soluble B vitamins is folic acid (synthetic *Lactobacillus casei* factor). The recognition of its function in the animal organism and its final synthesis<sup>1</sup> were the result of widely independent investigations in several laboratories extending over a period of fifteen years or more. Factors found essential for the growth of microorganisms (*L. casei* and *Streptococcus lactis R*) and factors found essential for the nutrition of the chick ( $B_c$ ) and of the monkey (vitamin M) may prove to have common physiologic significance.

Snell and Peterson<sup>2</sup> in 1940 found that a yeast extract and a solubilized liver fraction were good sources of a growth factor essential for *L. casei*. Mitchell and associates<sup>3</sup> in 1941 first used the term "folic acid" to describe a factor which they obtained from spinach and which is essential for the growth of *Str. lactis R*. Liver, yeast, and other substances proved to be rich sources of this factor. Hogan and Parrott<sup>4</sup> in 1939 used the term "vitamin  $B_c$ " to designate a dietary factor, obtained from a water-soluble extract of liver, which prevented macrocytic hyperchromic anemia in chicks. Pfiffner and co-workers<sup>5</sup> in 1943 isolated a crystalline compound from liver, and Campbell and associates<sup>6</sup> in 1944 showed that this compound would prevent nutritional macrocytic anemia in chicks. The designation of Hogan and Parrott (vitamin  $B_c$ ) was retained. The term "vitamin M" was employed by Day and associates<sup>7</sup> in 1938 to designate that factor in yeast and in liver which protected monkeys from fatal dietary cytopenia. In 1945 Day and co-workers<sup>8</sup> expressed the belief that *L. casei* factor is identical with vitamin M.

On the recognition that substances so widely scattered in nature are essential growth factors and possess antianemic potencies, numerous reports of the influence of liver and yeast concentrates, known to contain *L. casei* factor, were reported. Experimental anemia was induced in various ways. Spicer and associates<sup>9</sup> in 1942 reported that the leukopenia induced in rats by sulfonamide drugs could be prevented with liver extracts containing this factor. Kornberg and associates<sup>10</sup> in 1943 were able to correct severe granulocytopenia. By means of a folic acid concentrate Ransone and Elvehjem<sup>11</sup> in 1943 increased the leukocyte production in rats given sulfasuxidine. Likewise, Axelrod and co-workers<sup>12</sup> in 1943 corrected experimental leukopenia with a norite eluate factor or with whole liver. Higgins<sup>13</sup> in 1944 reported that a vitamin  $B_c$  concentrate had a marked antianemic effect on rats given promin.

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After the isolation of vitamin B<sub>12</sub> by Pfaffner and associates<sup>5</sup> in 1943 and of the *L. casei* factor by Hutchings and co-workers<sup>14</sup> in 1944, additional studies using purified products were reported. Using crystalline folic acid, Daft and Sebrell<sup>15</sup> in 1943 reported the correction of experimental granulocytopenia. Mallory and associates<sup>16</sup> in 1944 increased granulocyte percentages in rats made leukopenic with succinylsulfathiazole. Spontaneous granulocytopenia observed in a small percentage of rats fed purified diets was corrected by the *L. casei* factor (Kornberg and co-workers<sup>17</sup> in 1945). Likewise, *L. casei* factor was found by Kornberg and co-workers<sup>18</sup> in 1944 to have a preventive as well as corrective action on induced hemorrhagic anemia. Granulocytopenia induced by thiourea, when thyroxine or powdered thyroid was given simultaneously, was found by Daft and associates<sup>19</sup> in 1946 to be corrected by crystalline *L. casei* factor. But neutropenia induced by the injection of benzol, a bone marrow depressant, was not corrected by folic acid, according to Platt and co-workers<sup>20</sup> in 1946. The neutropenia following administration of sulfonamide compounds was considered to be due to a decrease of the number of coliform organisms which presumably synthesize the *L. casei* factor in the lower part of the intestinal tract. Exogenous folic acid thus was able to correct the induced neutropenia.

The extensive literature covering the reports of studies on the effects of *L. casei* factor on various cases of clinical anemia was reviewed by Berry and Spies<sup>21</sup> in 1946. Synthetic *L. casei* factor appeared to exert striking antianemic effects on the several types of macrocytic anemia and was found effective in inducing remissions in some cases of pernicious anemia. Goldsmith,<sup>22</sup> reporting late in 1946 her study of five patients who had severe anemia, stated that there was improvement in their physical condition as well as in their hematologic status on the administration of *L. casei* factor. Bethell and collaborators<sup>23</sup> in 1946 stated that therapeutic effects were not obtained when the B<sub>12</sub> conjugate was given to a gastrectomized patient, but an equivalent amount of folic acid incited a significant hematologic response.

Experimental gastrectomy has been shown to induce marked hematologic changes in some species of animals. The literature is extensive, and space does not permit a comprehensive review of the available reports. Briefly, the total removal of the stomach from rats (Jung,<sup>24</sup> 1933; Maisson and associates,<sup>25</sup> 1933; Bussabarger and Jung,<sup>26</sup> 1936; and Ivy,<sup>27</sup> 1940) induced changes of body weight and severe hematologic changes including marked microcytosis. Large amounts of ferric ammonium citrate did not alter the weight curve but did improve the blood picture strikingly. A complete return to normal levels was difficult to attain with iron alone, but the administration of a concentrate of a liver protein with iron was reported to correct the hematologic dyscrasia. It was obvious that the stomach is an essential organ for the maintenance of health in the rat, but blood findings in any way resembling those of pernicious anemia did not ensue on its removal.

Since synthetic *L. casei* factor has been shown to be corrective for certain clinical anemias, and since it has been shown to be effective in controlling cer-

tain nutritional anemias and other experimental anemias thought to be induced by altering intestinal flora by various drugs, a study was made of its effectiveness in correcting the blood changes incident to the removal of the stomach.

### EXPERIMENTAL PROCEDURES

Healthy adult male rats of the Sprague-Dawley strain were selected. Gastrectomy was performed with the rats under ether anesthesia, the esophagus being joined to the duodenum by an end-to-side anastomosis. Sixteen animals were gastrectomized; eight were not submitted to operation and served as the control group. All animals, both control and test, were provided a purified diet consisting of 64 per cent sucrose, 24 per cent vitamin-free casein (Labco brand), 6 per cent corn oil, 2 per cent cod-liver oil, and a 4 per cent salt mixture No. 2, U.S.P. XII. Adequate amounts of thiamine, riboflavin, pyridoxine, niacin, calcium pantothenate, choline, inositol, and para-amino-benzoic acid were supplied. Water was provided ad libitum. All animals were maintained in metal cages, on metal screen three mesh to an inch, three animals to a cage. The average food intake was recorded daily for three two-week periods: immediately after operation, two months after operation, and seven months after operation. Animals were weighed at intervals of two weeks.

Samples of blood, always obtained from the hearts of both control and test animals, were examined at three weeks, six weeks, three months, and five months after gastrectomy. Shortly after the five-month sampling, 200 micrograms of synthetic *L. casei* factor\* were given to each control and test animal by stomach tube. Seventy-two hours later the blood was again examined and data were assembled.

Two weeks later when the effects of the previous administration were presumably gone, the animals were fed the purified diet to which *L. casei* factor was added at a level of 100 micrograms per gram. After fourteen days on this dietary regimen the blood was again sampled and the data were assembled.

The purified diet, without the *L. casei* supplement, was again provided to the animals. After an interval of time sufficient to free the animals of the orally administered vitamin, all gastrectomized rats were given, each day for fourteen days, 400 micrograms of *L. casei* factor, intraperitoneally. At the end of this time the blood was again sampled and corresponding data were assembled.

### RESULTS

All animals withstood the immediate postoperative period reasonably well. Three died of obstruction at the site of the anastomosis within the first two weeks. Three died as a result of hemopericardium occasioned by heart puncture, and four died during the summer for reasons presumably unrelated to the study. In cases of obstruction an abscess usually developed at the site of anastomosis, and the body weight of the animal dropped rapidly. Only the six gastrectomized animals which survived the initial five-month period and were without obvious pathologic defects were used in the computation of the following data.

*A. Food Intake.*—In the initial period, soon after gastrectomy, control animals ate on the average 15.1 Gm. (63.4 calories) of the diet per day, while the gastrectomized animals took 12.7 Gm. (52.3 calories) per day. Two months later, the nongastrectomized control animals were eating 10.7 Gm. (44.9 calories), while the gastrectomized animals were eating approximately an equal amount, 10.3 Gm. (43.2 calories) per day. This period of measuring the food intake was in midsummer, and it is our belief that the decline in the amount

\*The pteroylglutamic (synthetic folic) acid employed in this study was furnished by Dr. Stanton M. Hardy of the Lederle Laboratories, Inc., Pearl River, N. Y.



consumed by the controls may have been owing to the higher temperatures and to the increased humidity levels of the season. Seven months after the operation, the control animals were eating on the average 11.7 Gm. (49.1 calories), while the gastrectomized animals were taking 8.9 Gm. (37.4 calories) daily. Although the food intake appeared to be quite satisfactory, only slightly less being consumed by the gastrectomized animals than by their controls; yet, the gastrectomized animals appeared undernourished, their coats were rough and unkempt, and all showed marked evidence of nutritional deficiencies.

*B. Body Weights.*—The average weight of the eight nongastrectomized control animals at the beginning of the experiment was 320 grams; that of the sixteen gastrectomized animals, constituting the test group, was 310 grams. The body weight data of the test group given in Table I were obtained from the six gastrectomized animals which had survived for five months and which were continuously free of any obvious pathologic defect.

TABLE I. BODY WEIGHTS IN GRAMS

	CONTROL	TEST
Preoperative	320	310
Postoperative		
One week	337	296
Two weeks	352	305
One month	364	318
Two months	380	324
Five months	372	236
Seven months	335	207

It is obvious from these data that the gastrectomized animals lost weight for a brief period and then increased it slightly for two months after the operation. Their weight then fell rapidly, a third of their preoperative weight having been lost at seven months. This loss may be owing to the fact that their food intake at this period was also nearly a third less than the amounts consumed daily during the early period after gastrectomy.

*C. Effect on the Blood of a Single Oral Administration of Synthetic L. Casei Factor.*—Data assembled from the heart bloods of normal and gastrectomized rats, five months after operation, before, and seventy-two hours after giving 200 micrograms of *L. casei* factor by mouth are condensed in Tables II

TABLE II. BLOOD DATA FIVE MONTHS AFTER GASTRECTOMY

	ANIMALS	ERYTHRO- CYTES (MILLIONS PER C.M.M.)	ERYTHRO- CYTES (VOLUME IN CUBIC MICRONS)	HEMOGLO- BIN (GM. PER 100 G.C.)	LEUKO- CYTES (THOU- SANDS PER C.M.M.)	RETICULO- CYTES (PER CENT)
<i>Before Giving L. casei Factor</i>						
Control	4	7.96 ± 0.2*	53.4 ± 2.5	14.3 ± 0.5	11.8 ± 1.1	1.3 ± 0.1
Gastrectomized	6	8.96 ± 0.1	38.9 ± 1.3	9.0 ± 0.8	10.7 ± 1.5	4.1 ± 0.4
<i>Seventy-Two Hours After Giving 200 Micrograms of Synthetic L. casei Factor Orally to Control and Gastrectomized Animals</i>						
Control	4	8.60 ± 0.3	46.4 ± 2.2	13.7 ± 0.2	9.8 ± 0.7	3.6 ± 0.6
Gastrectomized	6	9.30 ± 0.2	35.5 ± 0.5	8.6 ± 0.7	10.8 ± 1.1	6.4 ± 0.7

\*Probable error of the mean.

TABLE III. BLOOD DATA FIVE MONTHS AFTER GASTRECTOMY

	Differential distribution (per cent)							TOTAL NEUTRO- PHILS (THOU- SANDS PER C.M.M.)
	LYMPHO- CYTES	MONO- CYTES	GRANULOCYTES					
			EOSINO- PHILS	BASO- PHILS	NEUTROPHILS			
					MYELO- CYTES	METAMYE- LOCYTES	MATURE	
Before Giving <i>L. casei</i> Factor								
Control	60.0 ± 3.9*	0.5	3.5 ± 0.5	0.4	3.7 ± 0.6	4.2 ± 0.9	27.7 ± 2.7	4.2 ± 0.5
Gastrec- tomized	47.6 ± 1.5	0.6	0.6	0	6.2 ± 0.5	2.8 ± 0.6	42.2 ± 2.2	5.6 ± 0.9
Seventy-Two Hours After Giving 200 Micrograms of Synthetic <i>L. casei</i> Factor Orally								
Control	53.6 ± 3.9	1.7	3.5 ± 0.5	0.2	9.0 ± 0.8	2.0 ± 0.4	30.0 ± 2.8	3.4 ± 0.1
Gastrec- tomized	44.3 ± 2.0	0.5	0.1	0	11.2 ± 0.7	3.6 ± 0.6	40.3 ± 2.5	5.8 ± 0.5

\*Probable error of the mean.

and III. Healthy gastrectomized animals never had low total erythrocyte counts. The mean of the data obtained from thirteen animals three weeks after operation was  $8,550,000 \pm 200,000$  cells per cubic millimeter of blood. At that time their erythrocyte volumes and their hemoglobin levels did not differ from those values found in the nongastrectomized control animals. At five months, however, there was significant microcytosis and the amount of hemoglobin in grams per 100 c.c. of blood was greatly reduced.

A single administration of *L. casei* factor was without significant effect on the total erythrocyte count, the erythrocyte volumes, the grams of hemoglobin, or the total leukocyte count. Slightly significant elevations, however, were obtained in the reticulocyte percentages. In the data on leukocytes we observed some increase of the percentages of myelocytes in the blood of both control and test animals, but the total number of neutrophilic leukocytes was not increased. The conclusion was indicated that a single oral administration of the factor, in the amounts given, was without significant effect on the blood, seventy-two hours later.

*D. Effect on the Blood of Including Synthetic L. Casei Factor in the Diet, at a Level of 100 Micrograms Per Gram.*—Data assembled before placing animals on the diet, supplemented by *L. casei* factor, and those assembled after feeding this diet for two weeks are condensed in Tables IV and V. Although control animals took an average of 1,160 micrograms of the factor daily and test animals took an average of 920 micrograms daily, significant changes were not observed in the total number of erythrocytes per cubic millimeter, the volumes of erythrocytes, or the hemoglobin levels in gastrectomized animals. Again, there was a significant increase in the reticulocyte percentages in the test series. Although the percentage of lymphocytes in the blood of test animals dropped from 61.6 to 30.7, the total number of lymphocytes remained unchanged as a result of taking large amounts of the factor for fourteen days.

A marked increase, however, occurred in the percentage and in the total number of immature and mature neutrophilic granulocytes. These increments

TABLE IV. BLOOD DATA

	ANIMALS	ERYTHRO- CYTES (MILLIONS PER C.M.M.)	ERYTHRO- CYTES (VOLUME IN CUBIC MICRONS)	HEMOGLO- BIN (GM. PER 100 C.C.)	LEUKO- CYTES (THOU- SANDS PER C.M.M.)	RETICULO- CYTES (PER CENT)
<i>Prior to Giving Synthetic L. casei Factor</i>						
Control	3	8.43 ± 0.3*	50.5 ± 0.2	11.4 ± 0.2	8.0 ± 0.6	2.3 ± 0.3
Gastrec- tomized	5	8.46 ± 0.2	36.9 ± 1.7	6.9 ± 0.3	5.2 ± 0.7	3.2 ± 0.3
<i>Two Weeks Later: Synthetic L. casei Factor in Diet at Level of 100 Micrograms per Gram; Average Daily Intake: Controls, 1,160 Micrograms and Gastrectomized Animals, 920 Micrograms</i>						
Control	3	8.53 ± 0.1	50.4 ± 3.8	15.1 ± 0.6	9.8 ± 1.3	1.6 ± 0.1
Gastrec- tomized	5	7.92 ± 0.5	39.3 ± 0.8	7.3 ± 0.6	10.8 ± 1.2	5.9 ± 0.7

\*Probable error of the mean.

TABLE V. BLOOD DATA

DIFFERENTIAL DISTRIBUTION (PER CENT)								TOTAL NEUTRO- PHILS (THOU- SANDS PER C.M.M.)
LYMPHO- CYTES	MONO- CYTES	GRANULOCYTES						
		EOSINO- PHILS	BASO- PHILS	MYELO- CYTES	NEUTROPHILS			
					META- MYELO- CYTES	MATURE		
<i>Prior to Giving Synthetic L. casei Factor in Diet</i>								
Control	66.6 ± 6.3	1.5 ± 0.1	3.3 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	2.0 ± 0.2	21.6 ± 4.8	2.4 ± 0.7
Gastrec- tomized	61.6 ± 3.1	0.8	0	0	3.4 ± 0.2	1.2 ± 0.1	33.0 ± 1.1	1.9 ± 0.2
<i>Two Weeks Later: Synthetic L. casei Factor in Diet at Level of 100 Micrograms per Gram; Average Daily Intake: Controls, 1,160 Micrograms and Gastrectomized Animals, 920 Micrograms</i>								
Control	50.0 ± 0.4	0	3.5 ± 0.1	0	7.5 ± 1.6	3.5 ± 0.9	35.5 ± 2.1	4.6 ± 0.6
Gastrec- tomized	30.7 ± 1.7	0	1.6 ± 0.1	0	10.0 ± 0.4	4.0 ± 1.1	53.7 ± 2.3	7.2 ± 0.7

\*Probable error of the mean.

TABLE VI. BLOOD DATA AFTER GIVING SYNTHETIC L. CASEI FACTOR (400 MICROGRAMS)  
INTRAPERITONEALLY DAILY FOR FOURTEEN DAYS TO GASTRECTOMIZED ANIMALS

ERYTHROCYTES (MILLIONS PER C.M.M.)	ERYTHROCYTES (VOLUMES IN CUBIC MICRONS)	HEMOGLOBIN (GM. PER 100 C.C.)	LEUKOCYTES (THOUSANDS PER C.M.M.)	RETICULOCYTES (PER CENT)			
8.30 ± 0.3*	38.3 ± 1.0	7.0 ± 0.8	14.5 ± 1.3	6.1 ± 1.2			
DIFFERENTIAL DISTRIBUTION (PER CENT)							
LYMPHO- CYTES	MONO- CYTES	GRANULOCYTES			TOTAL NEUTROPHILS (THOUSANDS PER C.M.M.)		
		EOSINO- PHILS	BASO- PHILS	NEUTROPHILS			
				MYELO- CYTES		META- MYELO- CYTES	MATURE
21.6 + 2.1	0.2	2.0 ± 0.1	0	22.6 ± 3.4	7.0 ± 1.0	46.6 ± 4.4	11.1 ± 1.1

\*Probable error of the mean.

were more marked in test animals than in the controls. Percentages of myelocytes increased from 3.4 to 10.0; of metamyelocytes, from 1.2 to 4.0; and of mature granulocytes, from 33.0 to 53.7. Thus, as a result of providing the *L. casei* factor in the diet for fourteen days, myeloid stimulation produced an absolute increase of total circulating neutrophilic granulocytes from 1,900 to 7,200 per cubic millimeter of blood.

*E. Effect on the Blood of Gastrectomized Rats of Injecting 400 Micrograms of Synthetic L. Casei Factor Intraperitoneally Daily for Fourteen Days.*— Since we wished to avoid the risk of losing valuable animals, data on the blood were not assembled prior to the intraperitoneal injections of *L. casei* factor. Two weeks had elapsed, however, since the conclusion of the preceding experiment; so that we had reason to believe that the blood picture had reverted to levels previously recorded. The data assembled after fourteen daily injections are condensed in Table VI.

Parenteral administration of the factor was without effect on the total erythrocyte count, the size of erythrocytes, or the grams of hemoglobin per 100 c.c. of blood. There was a slightly greater increase in the percentage of reticulocytes and a considerably greater increase in the total number of leukocytes than occurred when more than twice the amount of the factor was taken with the diet for the same length of time. Again, the total number of lymphocytes per cubic millimeter of blood remained unchanged as a result of these daily injections. However, the percentages of myelocytes and metamyelocytes were both significantly higher, and the total number of neutrophilic granulocytes per cubic millimeter of blood increased from the previous level of 1,900 to 11,100.

It seems obvious from our data that neither the oral nor the parenteral administration of synthetic *L. casei* factor to gastrectomized rats produced a significant effect on the total erythrocyte count, the volumes of erythrocytes, or the grams of hemoglobin per 100 c.c. of blood. It had a decided effect, however, on the myeloid series of leukocytes and its prolonged administration (two weeks) either parenterally or orally resulted in a marked increase of the numbers of immature and mature neutrophilic granulocytes in the circulating blood.

#### SUMMARY

A study is reported of the effects on the blood changes induced by gastrectomy in white rats, of giving synthetic *L. casei* factor. Sixteen adult male rats were gastrectomized. Of these, six survived for five months and were used as test animals. All were without obvious signs of any gross pathologic changes, as a result of the surgical intervention, but all showed evidence of malnutrition, manifested by progressive loss of weight, and by their rough and discolored coats. Eight nongastrectomized rats served as controls.

Five months after gastrectomy, the total erythrocyte counts were normal, the hemoglobin levels were reduced, and marked microcytosis had developed. Seventy-two hours after a single administration of synthetic *L. casei* factor (200 micrograms), a slight but significant elevation of the percentage of reticulocytes was obtained, but there were no changes in the total erythrocyte counts, the volumes of erythrocytes, the hemoglobin levels, or the total leukocyte counts.

When *L. casei* factor was added to the purified diet, at a level of 100 micrograms per gram, and fed to gastrectomized rats for fourteen days, changes did not occur in the total numbers of erythrocytes, the volumes of erythrocytes, or the levels of hemoglobin. Slightly significant increases occurred in the percentages of reticulocytes and markedly significant increases occurred in the total numbers of leukocytes. This increase of the number of leukocytes was due entirely to a myeloid stimulation, for the numbers of lymphocytes remained unchanged. There was a marked increase of the total number of circulating myelocytes, metamyelocytes, and mature neutrophilic granulocytes.

When *L. casei* factor was injected intraperitoneally daily for fourteen days in amounts equivalent to 400 micrograms, a marked myeloid stimulation likewise occurred. Of the total leukocytes present, 22.6 per cent were myelocytes, 7.0 per cent were metamyelocytes, and 46.6 per cent were mature neutrophilic granulocytes. Thus, of the total number of circulating leukocytes, 76.2 per cent were neutrophilic granulocytes. The parenteral administration of the factor was more effective than providing the factor in the diet.

#### REFERENCES

1. Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., Subbarow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr.: The Structure and Synthesis of the Liver *L. casei* Factor. *Science* 103: 667-669, 1946.
2. Snell, E. E., and Peterson, W. H.: Growth Factors for Bacteria. X. Additional Factors Required by Certain Lactic Acid Bacteria. *J. Bact.* 39: 273-283, 1940.
3. Mitchell, H. K., Snell, E. E., and Williams, R. J.: The Concentration of "Folic Acid," *J. Am. Chem. Soc.* 63: 2284, 1941.
4. Hogan, A. G., and Parrott, E. M.: Anemia in Chicks Due to Vitamin Deficiency, *J. Biol. Chem.* 128: 46-47, 1939.
5. Piffner, J. J., Binkley, S. B., Bloom, E. S., Brown, R. A., Bird, O. D., Emmett, A. D., Hogan, A. G., and O'Dell, B. L.: Isolation of the Antianemia Factor (Vitamin B<sub>12</sub>) in Crystalline Form From Liver, *Science* 97: 404-405, 1943.
6. Campbell, C. J., Brown, R. A., and Emmett, A. D.: The Role of Crystalline Vitamin B<sub>12</sub> in the Nutrition of the Chick, *J. Biol. Chem.* 154: 721-722, 1944.
7. Day, P. L., Langston, W. C., and Darby, W. J.: Failure of Nicotinic Acid to Prevent Nutritional Cytopenia in the Monkey, *Proc. Soc. Exper. Biol. & Med.* 38: 860-863, 1938.
8. Day, P. L., Mims, V., Totter, J. R., Stokstad, E. L. R., Hutchings, B. L., and Sloane, N. H.: The Successful Treatment of Vitamin M Deficiency in the Monkey With Highly Purified *Lactobacillus casei* Factor, *J. Biol. Chem.* 157: 423-424, 1945.
9. Spicer, S. S., Daft, F. S., Sebrell, W. H., and Ashburn, L. L.: Prevention and Treatment of Agranulocytosis and Leukopenia in Rats Given Sulfamylguanidine or Succinylsulfathiazole in Purified Diets, *Pub. Health Rep.* 57: 1559-1566, 1942.
10. Kornberg, A., Daft, F. S., and Sebrell, W. H.: Production and Treatment of Granulocytopenia and Anemia in Rats Fed Sulfonamides in Purified Diets, *Science* 98: 20-22, 1943.
11. Ransone, B., and Elvehjem, C. A.: The Value of Biotin, Folic Acid Concentrates, and Liver Extract in the Diet of Rats Fed Succinylsulfathiazole, *J. Biol. Chem.* 151: 109-115, 1943.
12. Axelrod, A. E., Gross, P., Bosse, M. D., and Swingle, K. F.: Treatment of Leucopenia and Granulopenia in Rats Receiving Sulfaguanidine in Purified Diets, *J. Biol. Chem.* 148: 721-722, 1943.
13. Higgins, G. M.: The Influence of Vitamin B<sub>12</sub> Concentrate (Folic Acid) on Experimental Anemia in the Rat, *Proc. Staff Meet., Mayo Clin.* 19: 329-336, 1944.
14. Hutchings, B. L., Stokstad, E. L. R., Bohonos, N., and Slobodkin, N. H.: Isolation of New *Lactobacillus casei* Factor, *Science* 99: 371, 1944.
15. Daft, F. S., and Sebrell, W. H.: The Successful Treatment of Granulocytopenia and Leukopenia in Rats With Crystalline Folic Acid, *Pub. Health Rep.* 58: 1542-1545, 1943.
16. Mallory, M. E., Mims, V., Totter, J. R., and Day, P. L.: Effect of a Concentrate of Potential Streptococcal Factor on Growth and White Blood Cell Counts of . . . *J. Biol. Chem.* 156: 317-321, 1944.

17. Kornberg, A., Daft, F. S., and Sebrell, W. H.: Dietary Granulocytopenia in Rats Corrected by Crystalline *L. casei* Factor ("Folic Acid"), *Proc. Soc. Exper. Biol. & Med.* 58: 46-48, 1945.
18. Kornberg, A., Tabor, H., and Sebrell, W. H.: The Effect of *L. casei* Factor ("Folic Acid") on Blood Regeneration Following Hemorrhage in Rats, *Am. J. Physiol.* 142: 604-614, 1944.
19. Daft, F. S., Kornberg, A., Ashburn, L. L., and Sebrell, W. H.: Granulocytopenia in Rats Given Thiourea and Thyroxin. The Therapeutic Effect of *L. casei* Factor. *Proc. Soc. Exper. Biol. & Med.* 61: 154-158, 1946.
20. Platt, W. R., Bluestein, S. G., and Sisson, R. G.: The Effect of Liver Extract and Methyl Acetamide With Para-chloro-xyleneol on Artificially Induced Leukopenia in Rats, *Yale J. Biol. & Med.* 18: 275-279, 1946.
21. Berry, L. J., and Spies, T. D.: The Present Status of Folic Acid, *Blood* 1: 271-306, 1946.
22. Goldsmith, G. A.: Synthetic *L. casei* Factor (Folic Acid) in Treating Certain Anemias in Man, *Science* 104: 439, 1946.
23. Bethell, F. H., Swendseid, M. E., Bird, O. D., Meyers, M. C., Andrews, G. A., and Brown, R. A.: Observations on the Utilization of Folic Acid (Vitamin B<sub>12</sub>) and Vitamin B<sub>12</sub> Conjugate by Persons With Pernicious Anemia and Macrocytic Anemia Following Gastrectomy, *Univ. Hosp. Bull., Ann Arbor* 12: 42, 1946.
24. Jung, F. T.: Anemia Produced by Gastrectomy in the Rat, *Tr. Am. Gastro-Enterol. A.* 1933, pp. 237-239.
25. Maison, G. L., Highstone, W. H., Mayka, J., Burgeri, P. H., and Murray, D. E.: Anemia Following Gastrectomy in the Rat, *Am. J. Physiol.* 105: 69-70, 1933.
26. Bussabarger, R. A., and Jung, F. T.: Dietary and Hematologic Studies After Gastrectomy in the Rat, *Am. J. Physiol.* 117: 59-67, 1936.
27. Ivy, A. C.: Effects of Gastrectomy in Animals, *Am. J. Digest. Dis.* 7: 500-502, 1940.

## PERNICIOUS ANEMIA AND SUSCEPTIBILITY TO GASTRIC NEOPLASMS

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WITHIN the past fifteen years, increasing attention has been drawn to the coexistence of pernicious anemia and epithelial tumors of the stomach. That this association is more than accidental has been demonstrated by a number of large-scale statistical studies of both post-mortem and living cases. Nevertheless, clinical recognition of this fact has been limited and efforts at utilization of pernicious anemia as an "indicator" for the detection of gastric carcinoma in its early stages have been all too few. For this reason, it was thought worthwhile to review the evidence on this subject anew and to discuss its significance in gastric cancer detection programs.

### STATISTICAL CONSIDERATIONS

The numerous individual case reports of coexisting addisonian anemia and gastric cancer have been collected by Cotti,<sup>10</sup> Kaplan and Rigler,<sup>28</sup> and others. These have little or no statistical value and will be omitted from this discussion. Similarly, reports of multiple cases of this type have been published by Jenner,<sup>26</sup> Strandell,<sup>31</sup> Cotti,<sup>10</sup> Murphy and Howard,<sup>41</sup> and others. In most of these the anemia had been present for years before the appearance of the gastric tumor, the longest interval being seventeen years in the case cited by Miller.<sup>33</sup> This observation was confirmed by Doehring and Eusterman,<sup>12</sup> who found that the average duration of pernicious anemia prior to the onset of symptoms attributable to the gastric cancer was 8.7 years in their series.

Comparison of the reports from the Mayo Clinic over a period of twenty-five years reveals a distinct increase in the incidence of gastric cancer in patients with pernicious anemia. In the first publication by Giffin and Bowler,<sup>17</sup> only one gastric carcinoma and four other malignant tumors were noted in a series of 628 cases. In the period from 1925 to 1930, Conner and Birkeland<sup>9</sup> found twenty cases of cancer of the stomach among 658 patients with pernicious anemia. Washburn and Rozendaal<sup>62</sup> collected eleven more such cases in the next three-year period. Finally, Doehring and Eusterman<sup>12</sup> reported seventeen additional cases in a series of 1,014 patients with pernicious anemia studied from 1935 to 1939. The greatly increased life expectancy of individuals with addisonian anemia since the introduction of liver therapy is generally offered as an explanation for this apparent increase in gastric cancer morbidity among them.

Not only cancer but adenomatous polyps of the stomach have frequently been reported in association with pernicious anemia.<sup>12, 19, 62</sup> Velde<sup>60</sup> found six such cases (14 per cent) in a series of forty-two patients with pernicious anemia,

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as contrasted with an incidence of 0.71 per cent in a control series of 1,271 patients with other diseases. Such observations assume added significance when the apparently precancerous nature of gastric polyps is considered. In 1930, Miller and associates<sup>39</sup> reported eight personal cases and twenty-four others from the literature in which adenomatous gastric polyps had developed into frank carcinomas. Stewart<sup>53</sup> found carcinoma associated with thirteen of forty-seven gastric polyps, and several other observations of similar character have since appeared.<sup>32, 35, 44</sup> Accordingly, the demonstration of a gastric polyp in association with pernicious anemia may be considered to approach in significance the demonstration of carcinoma *per se*.

These case reports, even in large series, are merely suggestive of a relationship between the two diseases. A statistical approach is essential to determine whether the frequency with which they are associated is greater than can reasonably be expected on the basis of chance alone. Several studies of this type have appeared, all of which have revealed a frequency of coexistence significantly greater than would have been predicted by probability.

Rambach<sup>43</sup> encountered 641 cases of gastric carcinoma and fifty of pernicious anemia in a series of 11,849 post-mortem examinations. In eleven cases the two diseases were present together, an incidence roughly five times greater than that due merely to chance. Jenner<sup>26</sup> noted five gastric carcinomas, four benign gastric tumors, and only one extragastric malignant neoplasm in a series of seventy-six autopsied cases of pernicious anemia. Brown<sup>5</sup> studied 151 cases of pernicious anemia in a total of 18,200 post-mortem examinations; twelve of these (8 per cent) had gastric polyps and one had a carcinoma of the stomach. The incidence of gastric polyps in the other autopsies is given as 0.003 per cent, which must be an error. However, even when the generally reported autopsy incidence of 0.5 per cent is used for comparison, the number of polyps in the pernicious anemia series is still significantly high.

In addition to the cases found at necropsy, some statistical studies on the morbidity of coexistent pernicious anemia and tumors of the stomach have appeared. Jenner<sup>26</sup> discovered eight cases of gastric cancer in 181 patients with pernicious anemia. He computed the expected morbidity of gastric cancer per decade among the living population of Amsterdam and found that the actual frequency in his pernicious anemia series was twelve times that in the "normal" population of comparable age. Van der Sande<sup>59</sup> similarly calculated morbidity statistics and likewise concluded that the two diseases were associated more often than chance alone would decree. Cotti<sup>10</sup> compiled all of the published case reports in which pernicious anemia had been associated with a malignant tumor and found that the stomach was the primary site in ninety-three of 107 cases, or 86 per cent of the entire series. Since cancer of the stomach represents only about 20 to 40 per cent of all cancers in the general living population, the marked predilection for this organ in patients with pernicious anemia is another link in the chain of corroboratory evidence.

In an attempt to eliminate certain possible sources of error in previous publications on the subject, Kaplan and Rigler<sup>28</sup> studied all cases of pernicious anemia, carcinoma of the stomach, and carcinoma of the colon, cecum, and rectum



recorded in a series of 43,021 consecutive autopsies performed by the Department of Pathology of the University of Minnesota. When corrections were applied for errors in the recorded diagnosis of pernicious anemia, an estimated total of 293 cases of this disease were available for study. Of these, thirty-six also had carcinoma of the stomach, seven had gastric polyps, and four had carcinoma of the colon, rectum, or cecum. Statistical analysis revealed that only twelve cases of carcinoma of the stomach would have been expected. The probability that the observed number was due to chance alone was less than one in one billion. It is apparent, therefore, from all of the foregoing evidence, that some etiologic relationship must exist between pernicious anemia and gastric epithelial tumors.

#### THEORETIC CONSIDERATIONS

The precise nature of this relationship, however, is still far from clear. On a purely hypothetic basis at least three possibilities can be enumerated: (1) Pernicious anemia directly produces a precancerous state in the stomach; (2) gastric cancer causes pernicious anemia; and (3) the two diseases are linked through a precursor or manifestation common to both.

It seems very unlikely that pernicious anemia is a direct cause of gastric tumors, although conclusive proof to this effect is lacking. That the disease is often in remission at the time when the cancer appears is a common observation, and Dyke and Harvey<sup>13</sup> observed several patients in whom adequate anti-pernicious anemia therapy for a number of years did not prevent the development of gastric carcinoma. The incidence of gastric cancer does not appear to be elevated in patients with other types of anemia, which suggests that anemia per se is not an important etiologic factor.

If cancer of the stomach were caused by exogenous agents in the diet, acting while in direct contact with the gastric mucosa, then the observation by Jacobson and Palmer<sup>25</sup> of prolonged gastric emptying time in patients with pernicious anemia in relapse might throw some light on the association of the two diseases. Although Roffo<sup>46</sup> has reported the induction of gastric tumors in rats fed heated fats or cholesterol, Kirby<sup>20</sup> and others have been unable to corroborate these results, and gastric carcinogens in the human diet are still hypothetic.

Cayer and associates<sup>6</sup> studied vitamin levels in patients with pernicious anemia and found that the levels of nicotinic acid, riboflavin, and thiamin were all significantly reduced. Although vitamins play a selective and important role in the experimental induction of liver cancer, their possible relation to gastric cancer development is entirely unknown.

Likewise, the possibility that gastric cancer causes pernicious anemia can be quickly disposed of by recalling that in many instances where the two have been associated, the latter disease was present for a number of years prior to the appearance of the neoplasm. Because of the frequent occurrence, in patients with gastric cancer, of macrocytic anemias superficially resembling pernicious anemia, some confusion on this point appears to have existed in former years.

In a recent hematologic survey of 127 patients with gastric cancer, Oppenheim and co-workers<sup>42</sup> found a state of anemia in seventy-eight, or 64 per cent.

The anemia was macrocytic in twenty-five cases, normocytic in thirty-five, and microcytic in eighteen. Leukopenia was absent in all but one, sternal marrow studies did not show failure of maturation and the other characteristic features of pernicious anemia, free hydrochloric acid was often present in the gastric secretions, and a typical reticulocyte response to intramuscular liver extract could not be elicited in some of these cases. Inasmuch as evidence of extensive hepatic insufficiency had previously been found in many patients with carcinoma of the stomach,<sup>1</sup> these authors were inclined to attribute the macrocytic anemias observed in their series to hepatic damage. No instance of true pernicious anemia was encountered in this study, a fact which seems at variance with the evidence previously presented. However, the incidence of pernicious anemia in other large series of gastric cancers, though significantly elevated, has still been only a few per cent. Moreover, in a number of personally observed cases, patients with treated pernicious anemia in remission developed microcytic anemias due to chronic hemorrhage following the appearance of their gastric cancers. In such cases the existence of an underlying pernicious anemia would have been completely masked except for the previous history. It is obvious, therefore, that in the series reported by Oppenheim and associates<sup>42</sup> the few cases of pernicious anemia that might ordinarily have been expected could well have been absent on a chance basis alone or obscured by the development of some complication such as chronic blood loss.

It is, of course, theoretically possible for a diffusely infiltrating cancer to destroy the entire gastric mucosa responsible for the formation of "intrinsic factor" and thus produce "secondary" pernicious anemia. Until recently, the absence of this complication in subtotally gastrectomized patients<sup>23</sup> was difficult to reconcile with the demonstration by Meulengracht<sup>36</sup> that the pyloric portion of the sheep stomach was the site of formation of intrinsic factor. This discrepancy has been resolved by the work of Fox and Castle,<sup>16</sup> who found that the human stomach differs from that of the sheep by the fact that the intrinsic factor is elaborated in the fundic rather than in the pyloric portion. Thus, gastrectomy would have to be *total*, including the entire cardia, in order to produce pernicious anemia as a sequel. In a recent review article, Bethell and co-workers<sup>2</sup> expressed the opinion that all patients surviving total gastrectomy for any prolonged period of time will develop pernicious anemia. Similarly, extensive cancers of the fundus and cardia of the stomach might conceivably cause secondary pernicious anemia, but such instances must be quite rare.

It is apparent, therefore, that the majority of cases in which the two diseases coexist must be explained in some other way. A number of features common to both conditions immediately come to mind as possible links between them. These factors include achlorhydria and achylia, atrophic gastritis, and heredity. A hypothetic diagram representing the possible relationships has been published by Rigler and associates.<sup>45</sup>

Haring<sup>19</sup> suggested that the functional deficiency of the gastric mucosa which results in achlorhydria and achylia also acts as a stimulus to proliferation and finally to neoplasia, in a manner analogous to the development of goiter and adenomas of the thyroid in iodine deficiency states. However, the frequency of

achlorhydria is far greater than that of gastric carcinoma, indicating that it is certainly not a dominant etiologic factor. Bloomfield and Pollard<sup>3</sup> closely followed a group of forty-three apparently healthy individuals with persistent histamine-refractory anaecidity for a number of years and observed neither gastric cancer nor pernicious anemia in any member of the group.

Gastritis has been considered an etiologic precursor of pernicious anemia.<sup>14, 22, 38</sup> However, Magnus and Ungley<sup>34</sup> carefully studied the stomachs of patients with pernicious anemia and found no inflammatory changes. Simple atrophy of the mucosa in the gastric fundus and corpus was the only consistent abnormality. It has been reported that on serial gastroscopic examination, the atrophy and "gastritis" seen in patients with pernicious anemia in relapse often disappears after adequate liver therapy.<sup>27</sup> It is not likely that an essential etiologic factor in a disease would disappear in this manner. Moreover, Moschcowitz<sup>40</sup> has pointed out that achlorhydria and achylia frequently exist for years in the absence of atrophic gastritis, the latter appearing only after the other manifestations of pernicious anemia become apparent. Thus, the weight of evidence would indicate that gastritis is not necessarily a causative factor in pernicious anemia.

The alternative possibility that gastritis is a precancerous lesion has been expounded for years by Konjetzny<sup>31</sup> and others. The frequent demonstration of gastritis in cancerous stomachs naturally suggests a causal relationship, and Warren and Meissner<sup>61</sup> have pointed out the histologic similarity between the severe epithelial changes in some cases of gastritis and the morphologic appearance of known precancerous lesions elsewhere. Torgerson<sup>58</sup> studied the distribution of gastric cancers in unselected cases and in patients with associated pernicious anemia and found that the proportion of tumors arising in the fundus was distinctly higher in the latter group. Because of the known tendency of mucosal atrophy in pernicious anemia to affect the fundus and corpus more than the pylorus, he felt that this distribution of tumors argued for the precancerous nature of atrophic gastritis. However, such evidence is only circumstantial; the careful studies of Hebbel<sup>29</sup> and Guiss and Stewart<sup>18</sup> have failed to demonstrate that gastritis is more common in cancerous stomachs than in those of other individuals of comparable age. Thus, although we cannot refute the possibility that chronic gastritis leads to gastric cancer, present evidence lends little or no support to this view.

The observation of familial tendencies in both pernicious anemia and gastric cancer indicates that common constitutional or hereditary factors may link the etiologies of the two diseases. Conner<sup>8</sup> first demonstrated the high incidence of achlorhydria in relatives of patients with pernicious anemia. Schwartz<sup>50</sup> reported that 18 per cent of a series of patients with pernicious anemia also had relatives with the disease. Among the numerous individual case reports in which pernicious anemia appeared in more than one generation of the same family are those of Faber and Gram<sup>15</sup> and MacLachlan and Kline.<sup>33</sup> The evidence for a familial component in the etiology of pernicious anemia has been summarized and discussed at length by Meulengracht<sup>37</sup> and by Moschcowitz.<sup>40</sup>

Pernicious anemia and gastric cancer have been reported separately or together in several members and in more than one generation of many families. The cases of Borovanská-Felklová<sup>4</sup> and Thiele<sup>57</sup> are particularly striking examples of this type. Zancan,<sup>63</sup> in a genealogic study of seventy-four patients with pernicious anemia, found evidence of nine carcinomas among their blood relatives, of which six arose in the stomach. He traced a parallel series of relatives of patients with gastric cancer and found that their incidence of gastric cancer was actually lower than that of the group related to patients with pernicious anemia. While such observations are not statistically conclusive, present evidence, though inadequate, favors the thesis that the two diseases are linked through common hereditary influences.

Ivy<sup>24</sup> recently suggested that a study of blood groups in patients with these coexisting diseases and in their blood relatives might yield further information along these lines. In his opinion, the fact that the gastric mucosa is a known storage site for the blood group-specific agglutinins may even have a direct bearing on the incidence of gastric cancer.

One additional hypothesis remains to be discussed. The increasing number of case reports of coexisting pernicious anemia and gastric cancer since the advent of liver therapy was attributed by Saltzman<sup>48</sup> to the longer survival period of patients with pernicious anemia, a view that has been generally accepted. However, Teuff<sup>56</sup> rejected this explanation and speculated that liver extract itself might contain a weak carcinogenic agent which on prolonged administration could lead to cancer in susceptible individuals. This rather startling suggestion finds some experimental support in the work of Kleinenberg and associates,<sup>30</sup> Steiner,<sup>52</sup> and several others who have shown that certain fractions of the livers of both cancerous and noncancerous individuals, when injected into mice or rats, will elicit malignant neoplasms at the site of injection in a small percentage of animals. It has been demonstrated by Strong and co-workers<sup>55</sup> that animals genetically selected for resistance to the local effects of a carcinogen may exhibit secondary susceptibility to the same agent at a remote site. In the light of such evidence, the possible carcinogenicity of therapeutic liver extracts may well deserve further study.

#### THE EARLY DETECTION OF GASTRIC CANCER

Recent advances in surgery have made possible the salvage of at least 25 per cent of patients with early, operable cancer of the stomach. Refinements in gastroscopic and roentgenologic technique have greatly improved the accuracy of diagnosis of such lesions. Were it not for the fact that early gastric cancer is generally asymptomatic, a considerable proportion of patients now dying of this condition could be saved. Thus, the chief problem in gastric cancer is becoming, more and more, its detection on a practical scale in asymptomatic individuals.

The most obvious way to attack this problem is to examine routinely, by roentgenologic or gastroscopic methods or both, all patients in the gastric cancer-bearing age. However, morbidity statistics compiled by Collins and associates<sup>7</sup> indicate that at any one time only three individuals per 1,000 in the

living population over 40 years of age would be likely to have a carcinoma of the stomach. On the basis of these figures, any mass survey in which no selection of patients was employed would entail an enormous expenditure of time, effort, and money with a very small yield of tumors. This prediction has been confirmed by the work of St. John and co-workers.<sup>47</sup> These investigators, using a rapid fluoroscopic technique, examined over 2,400 asymptomatic individuals past 50 years of age and discovered only two carcinomas and one lymphosarcoma of the stomach. More recently, Dailey and Miller<sup>48</sup> studied 500 apparently healthy men 45 years of age or over and encountered no malignant tumors of the stomach. Such results emphasize the necessity of *selecting* patients for routine periodic examinations of the stomach on the basis of some pre-existing disease, symptom, or other criterion with which the occurrence of gastric cancer can be shown to be associated more frequently than in the general population.

To date, the only known indicator of this type is pernicious anemia. Two years ago, Rigler and associates<sup>49</sup> presented the results of serial roentgenologic and gastroscopic examinations in a series of patients with this disease. At that time, 211 patients had been examined one or more times; of these, seventeen (8.0 per cent) were found to have carcinoma of the stomach, and fifteen (7.1 per cent) had benign polyps. Since then, forty-eight new cases of pernicious anemia have been examined at the University of Minnesota, yielding one additional gastric cancer and two individuals with benign polyps. The summarized data are given in Table I. Comparison of these results with the morbidity, previously cited, of gastric cancer in unselected cases of comparable age reveals clearly the striking "concentration" of carcinomas of the stomach in patients with pernicious anemia.

TABLE I. CLINICAL RESULTS

TOTAL NUMBER OF PATIENTS WITH PERNICIOUS ANEMIA EXAMINED	CARCINOMA OF STOMACH FOUND	BENIGN POLYPS OF STOMACH FOUND	TOTAL TUMORS OF STOMACH FOUND
259	18 (6.9%)	17 (6.6%)	35 (13.5%)

Although no large-scale program of gastric cancer detection has yet been established at New Haven Hospital, even a casual survey of patients with pernicious anemia followed for a reasonable period of time reveals that several have developed polyps, and carcinomas are known to have appeared in at least two such patients, one of whom is alive and well almost two years after gastrectomy.

Unfortunately, patients with pernicious anemia represent a very small fraction of those susceptible to gastric cancer. Other indicators must be determined by careful clinical studies on large groups of variously categorized patients. A large-scale investigation of this type is now in progress at the University of Minnesota, the preliminary results of which were recently reported by State and Wangenstein.<sup>51</sup> Achlorhydria, chronic gastritis, gall bladder disease, and anemia have all been used as criteria for the selection of

patients in this study, and relatives of patients with gastric cancer are also being serially examined. It is to be hoped that the final data will permit the nationwide establishment of selective gastric detection programs from which a high yield of early tumors may confidently be expected.

#### SUMMARY AND CONCLUSIONS

1. Statistical analyses reveal that pernicious anemia and carcinoma of the stomach develop in the same individuals more often than would be expected on the basis of chance alone and must, therefore, be etiologically related.

2. The evidence pertaining to the nature of this relationship is still inconclusive but suggests that the two diseases are probably linked indirectly through the medium of some common factor. Among the possible factors to be considered are hereditary or constitutional tendencies, achlorhydria, gastritis, and liver therapy.

3. In contrast to the paucity of gastric tumors discovered in nonselective mass surveys, serial gastric examination of individuals with pernicious anemia has yielded a very high incidence of benign and malignant epithelial tumors of the stomach.

4. It is suggested that a search for other conditions etiologically related to or associated with gastric cancer is a necessary prerequisite to the establishment of practical, *selective* mass surveys for the early detection of neoplasms of the stomach.

#### REFERENCES

1. Abels, J. C., Rekers, P. E., Binkley, G. E., Pack, G. T., and Rhoads, C. P.: Metabolic Studies in Patients With Cancer of Gastrointestinal Tract; Hepatic Dysfunction, *Ann. Int. Med.* 16: 221-240, 1942.
2. Bethell, F. H., Sturgis, C. C., Rundles, R. W., and Meyers, M. C.: Blood; a Review of Recent Literature, *Arch. Int. Med.* 76: 239-254, 1945.
3. Bloomfield, A. L., and Polland, W. S.: The Fate of People With Unexplained Gastric Anacidity, *J. Clin. Investigation* 14: 321-324, 1935.
4. Borovanská-Felklová: Quoted by Kohn, E., and Reimann, F.: Hämatologische Arbeiten aus der tschechischen Literatur, *Folia haemat.* 40: 173-179, 1930.
5. Brown, M. R.: Pathology of Gastrointestinal Tract in Pernicious Anemia and Subacute Combined Degeneration of Spinal Cord; Study of 151 Autopsies, *New England J. Med.* 210: 473-477, 1934.
6. Cayer, D., Ruffin, J. M., and Perlzweig, W. A.: Vitamin Levels in Pernicious Anemia, *Am. J. M. Sc.* 212: 179-184, 1946.
7. Collins, S. D., Gover, M., and Dorn, H. F.: Trend and Geographic Variation in Cancer Mortality and Prevalence, With Special Reference to Gastric Cancer, *J. Nat. Cancer Inst.* 1: 425-450, 1941.
8. Conner, H. M.: Hereditary Aspect of Achlorhydria in Pernicious Anemia; Study of Gastric Acidity in 154 Relatives of 109 Patients Having Pernicious Anemia, *Proc. Staff Meet., Mayo Clin.* 4: 200-202, 1929.
9. Conner, H. M., and Birkeland, I. W.: Coexistence of Pernicious Anemia and Lesions of Gastrointestinal Tract; Carcinoma of Stomach; Consideration of 20 Cases; 11 Reported, *Ann. Int. Med.* 7: 89-104, 1933.
10. Cotti, L.: Rilievi Casistico-Bibliografici e Contributo Personale in Merito Alla Conoscenza dei Rapporti Fra Anemia Perniciosa e Carcinoma Gastrico, *Haematologica* 19: 939-982, 1938.
11. Dailey, M. E., and Miller, E. R.: A Search for Symptomless Gastric Cancer in 500 Apparently Healthy Men of Forty-Five or Over, *Gastroenterology* 5: 1-4, 1945.
12. Doehring, P. C., and Eusterman, G. B.: Association of Pernicious Anemia and Carcinoma of the Stomach, *Arch. Surg.* 45: 554-563, 1942.
13. Dyke, S. C., and Harvey, E.: Efficient Treatment of Pernicious Anemia, *Lancet* 2: 59-62, 1933.
14. Faber, K.: Gastritis und Anämie, *Zentralbl. f. inn. Med.* 53: 1016-1026, 1932.

15. Faber, K., and Gram, H. C.: The Association of Achylia and Anemia of Different Types in Three Members of the Same Family, and the Behavior of the Color Index in Pernicious Anemia, *Arch. Int. Med.* 34: 827-832, 1921.
16. Fox, H. J., and Castle, W. A.: Observations on Etiologic Relationship of Achylia Gastrica to Pernicious Anemia; Difference in Site of Secretion of Intrinsic Factor in Dog and in Human Stomach, *Am. J. M. Sc.* 203: 18-28, 1942.
17. Giffin, H. Z., and Bowler, J. P.: Diseases Which May Be Associated With Pernicious Anemia, *Minnesota Med.* 6: 13-16, 1923.
18. Guise, L. W., and Stewart, F. W.: Chronic Atrophic Gastritis and Cancer of the Stomach, *Arch. Surg.* 46: 823-843, 1913.
19. Haring, W.: Magenpolypen und perniziös-anämisches Syndrom, *Fortschr. a. d. Geb. d. Röntgenstrahlen* 45: 521-531, 1932.
20. Hebbel, R.: Chronic Gastritis; Its Relation to Gastric and Duodenal Ulcer and to Gastric Carcinoma, *Am. J. Path.* 19: 43-71, 1943.
21. Hurst, A. F.: Achlorhydria and Achylia Gastrica, and Their Connexion with Addison's Anemia-Subacute Combined Degeneration Syndrome and Simple (Non-Addisonian) Achlorhydric Anaemia, *Quart. J. Med.* 1: 157-177, 1932.
22. Hurst, A. F.: Gastritis, *Guy's Hosp. Gaz.* 50: 227-237, 1936.
23. Ingelfinger, F. J.: Late Effects of Total and Subtotal Gastrectomy, *New England J. Med.* 231: 321-327, 1944.
24. Ivy, A.: Discussion at Third Conference on Gastric Cancer, Chicago, December, 1946, *J. Nat. Cancer Inst.* To be published.
25. Jacobson, L. O., and Palmer, W. A.: The Effect of Anemia on Gastric Emptying, *Gastroenterology* 1: 1133-1140, 1943.
26. Jenner, A. W. F.: Perniziöse Anämie und Magenkarzinom, *Acta med. Scandinav.* 102: 529-590, 1939.
27. Jones, C. M., Benedict, E. B., and Hampton, A. D.: Variations in Gastric Mucosa in Pernicious Anemia; Gastroscopic, Surgical, and Roentgenologic Observations, *Am. J. M. Sc.* 190: 596-610, 1935.
28. Kaplan, H. S., and Rigler, L. G.: Pernicious Anemia and Carcinoma of the Stomach; Autopsy Studies Concerning Their Inter-relationship, *Am. J. M. Sc.* 209: 339-348, 1945.
29. Kirby, A. H. M.: Attempts to Induce Stomach Tumors; Effects of Cholesterol Esters Heated to 300° C., and of Cholesterol Heated to 430° C., *Cancer Research* 5: 129-134, 1945.
30. Kleinenberg, H. E., Neufach, S. A., and Schabad, L. M.: Endogenic Blastogenic Substances, *Am. J. Cancer* 39: 463-488, 1940.
31. Konjetzny, G.: Das Krankheitsbild der Gastro-Duodenitis, *Med. Klin.* 32: 473-475, 1936.
32. Lawrence, J. C.: Gastrointestinal Polyps; Statistical Study of Malignancy Incidence, *Am. J. Surg.* 31: 499-505, 1936.
33. MacLachlan, W. W. G., and Kline, F. M.: Occurrence of Anemia in Four Generations, *Am. J. M. Sc.* 172: 533-543, 1926.
34. Magnus, H. A., and Ungley, C. C.: The Gastric Lesion in Pernicious Anemia, *Lancet* 1: 420-421, 1938.
35. McRoberts, J. W.: Gastric Polyps, *Proc. Staff Meet., Mayo Clin.* 8: 685-690, 1933.
36. Meulengracht, E.: Histologic Investigation Into Pyloric Gland Organ in Pernicious Anemia, *Am. J. M. Sc.* 197: 201-214, 1939.
37. Meulengracht, E.: Heredity Factor in Pernicious Anemia, *Am. J. M. Sc.* 169: 177-195, 1925.
38. Miller, T. G.: Addisonian Anemia and Carcinoma of the Stomach in Same Individual; Report of Three Cases: Chronic Gastritis as Probable Basis for Both Diseases, *Internat. Clin.* 1: 167-181, 1935.
39. Miller, T. G., Lhason, E. L., and Wright, V. W. M.: Carcinomatous Degeneration of Polyp of Stomach; Report of 8 Personal Cases With Review of 24 Recorded by Others, *Arch. Int. Med.* 46: 841-878, 1930.
40. Moschowitz, E.: The Relation of Achlorhydria to Pernicious Anemia, *Arch. Int. Med.* 48: 171-186, 1931.
41. Murphy, W. P., and Howard, I.: Analysis of Complications Occurring in a Series of Patients With Pernicious Anemia, *Rev. Gastroenterol.* 3: 98-110, 1936.
42. Oppenheim, A., Abels, J. C., Pack, G. T., and Rhoads, C. P.: Metabolic Studies in Patients With Cancer of Gastrointestinal Tract; Anemia of Patients With Gastric Carcinoma, *J. A. M. A.* 127: 273-276, 1945.
43. Rambach, H.: Ueber die Entwicklung von Magenkrebs bei der perniziösen Anämie, *Monatschr. f. Krebsbekämpfung* 4: 201-213, 1936.
44. Rigler, L. G., and Erickson, L. G.: Benign Tumors of Stomach; Observations on Their Incidence and Malignant Degeneration, *Radiology* 26: 6-15, 1936.
45. Rigler, L. G., Kaplan, H. S., and Fink, D. L.: Pernicious Anemia and the Early Diagnosis of Tumors of the Stomach, *J. A. M. A.* 128: 426-432, 1945.

46. Roffo, A. H.: *Pirólisis de Colesterol; Alquitrán Cancerígeno del Colesterol*, Bol. Inst. de med. exper. para el estud. y trat. d. cáncer 18: 929-948, 1941.
47. St. John, F. B., Swenson, P. C., and Harvey, H. D.: *Experiment in the Early Diagnosis of Gastric Carcinoma*, Ann. Surg. 119: 225-231, 1944.
48. Saltzman, F.: *Perniziöse Anämie und Karzinom*, Acta med. Scandinav. 75: 198-204, 1931.
49. Schindler, R.: *Gastric Mucosa in Benign Adenomas*, Am. J. Digest. Dis. 9: 149-150, 1942.
50. Schwartz, S. O.: *Pernicious Anemia*, M. Clin. North America 28: 214-231, 1944.
51. State, D., and O. H.: *Paper presented at Third Conference on Gastric Cancer*, 1946, J. Nat. Cancer Inst. To be published.
52. Steiner, P. E.: *Cancerogenic Tissue Extract From Human Sources*, Science 92: 431-432, 1940.
53. Stewart, M. J.: *Observations on Relation of Malignant Disease to Benign Tumours of the Intestinal Tract*, Brit. M. J. 2: 567-569, 1929.
54. Strandell, B.: *Pernicious Anemia; Study of 117 Cases*, Acta med. Scandinav. (Suppl.) 40: 1-124, 1931.
55. Strong, L. C., Collins, V. J., and Durand, E. A.: *Genetic Analysis of Induction of Tumors by Methylcholanthrene; Probable Remote Induction of Various Types of Gastric Lesions*, Cancer Research 3: 21-28, 1943.
56. Teuff, R.: *Perniziöse Anämie und Magenkarzinom*, Arch. f. Verdauungskr. 61: 166-180, 1937.
57. Thiele, W.: *Perniziöse Anämie und Magenkarzinom unter besonderer Berücksichtigung ihres familiären Auftretens*, Klin. Wchnschr. 15: 921-923, 1936.
58. Torgerson, J.: *Localization of Gastritis and Gastric Cancer, Especially in Cases of Pernicious Anemia*, Acta radiol. 25: 845-855, 1944.
59. van der Sande, D.: *Question of Relation Between Pernicious Anemia and Gastric Cancer*, Nederl. tijdschr. v. geneesk. 80: 4774-4781, 1936.
60. Velde, G.: *Die Beziehungen zwischen perniziöser Anämie, Magenpolypen, und Magenkarzinom*, Ztschr. f. klin. Med. 134: 653-670, 1938.
61. Warren, S., and Meissner, W. A.: *Chronic Gastritis and Carcinoma of the Stomach*, Gastroenterology 3: 251-256, 1944.
62. Washburn, R. N., and Rozendaal, H. M.: *Gastric Lesions Associated With Pernicious Anemia*, Ann. Int. Med. 11: 2172-2180, 1938.
63. Zancan, B.: *Sui rapporti tra anemia perniziosa e carcinoma gastrico*, Minerva med. 2: 645-650, 1937.



## THE RADIOSENSITIVITY OF ERYTHROBLASTS

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**A**LTHOUGH it is generally agreed that the bone marrow may be made extremely aplastic as a result of irradiation, opinion has differed as to the relative sensitivities of the various cell lineages. The attention of most investigators seems to have been directed to a study of the effect of irradiation on the granulocyte precursors. Heineke (1903, 1905), in his classical paper on the effects of x-rays on bone marrow, saw clearly the destruction of myelopoiesis but hardly considered the question of the effect of irradiation on erythropoiesis. However, the great numbers of degenerating lymphocytes which he described were probably degenerating erythroblasts. A number of investigators, including Milechner and Mosse (1904), Aubertin and Beaujard (1905), and Jolly (1924), reported that erythroblasts are more resistant than the other myeloid cells. Krause and Ziegler (1906), Lacassagne and Lavedan (1924), Halberstaedter and Simons (1933), and den Hoed, Levie, and Straub (1938) found that after heavier or repeated treatment, the erythroblasts also show evidence of injury. The divergent opinions on the relative radiosensitivity of erythroblasts are reviewed by Selling and Osgood in Downey's *Handbook of Hematology* (1938). More recently, in Shields Warren's review (1942), Dunlap concludes: "Similarly, the erythropoietic tissue, according to the majority of observers, shows considerable initial radioresistance, but once it is damaged its ability to recover is limited."

Our own observations on the effect of radiation on the bone marrow of rabbits, rats, mice, and chickens have shown the erythropoietic cells to be among the most sensitive of all hematopoietic cells. The findings which have led us to this conclusion will be given briefly in this report. A more detailed presentation will be found in Chapter VI of our report on the "Histopathology of Irradiation From External and Internal Sources" in the Plutonium Project Reports.

### MATERIALS AND METHODS

The effects on bone marrow of total body irradiation by single doses of x-rays, fast and slow neutrons, and of radiations from internal sources (strontium<sup>90</sup>, barium<sup>140</sup>, lanthanum<sup>140</sup>, phosphorus<sup>32</sup>, sodium<sup>24</sup>, yttrium<sup>91</sup>, radium, and plutonium) were studied as part of the program of the Histology Group of the Health Division, Metallurgical Laboratory, University of Chicago. For most of the experiments the species used were mice and rats. In addition, a large number of chickens and rabbits were exposed to x-rays and two series of rabbits to fast neutrons. In general, the dosages used were the LD 50/30 days.\* Lower doses also were used, these being decreased until morphologic changes were no

From the Metallurgical Laboratory, University of Chicago, and the Argonne National Laboratory.

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\*The dose which kills 50 per cent of the animals within thirty days.

longer found. For each experiment a group of animals of the same strain and comparable weight and age was irradiated and sacrificed at predetermined intervals. Untreated controls were sacrificed at the same intervals.

X-rays of 200 kvp were filtered with 0.5 mm. of copper and 1.0 mm. of aluminum (Hagen and Zirkle, 1946). The animals were held in plastic boxes and were rotated at least once during the course of the exposure. The distance varied somewhat with the size and number of animals treated at one time, but it was between 70 and 90 cm., giving from 15 to 11 r per minute.

Fast neutrons were primarily from the Clinton pile (Zirkle and Raper, 1946). Some rabbits were exposed to fast neutrons from the University of Chicago cyclotron (Hagen and Zirkle, 1946).

Slow neutrons were given to mice in boron-lined lead boxes inserted in the Clinton pile tunnels (Zirkle and Raper, 1946).

Internally administered  $\beta$  and  $\gamma$  emitters included sodium<sup>24</sup>, barium<sup>140</sup>, lanthanum<sup>140</sup>, strontium<sup>90</sup>, and phosphorus<sup>32</sup> injected intraperitoneally, and yttrium<sup>91</sup> injected intravenously.

Among  $\alpha$  emitters, plutonium nitrate or citrate (Snyder and Finkle, 1946) was administered intravenously to mice and rats, or intramuscularly to mice. Radium from which the radon had been blown off twenty-four hours earlier (Norris, Tompkins, and Finkle, 1946) was given intraperitoneally to mice and rats.

Femurs of rats and mice were opened at autopsy by shaving nearly to the midline with a sharp knife, and the entire bone then was fixed in Zenker-formol. To secure the bone marrow of rabbits and chickens, the bone was chipped from the shaft of the femur and a pencil of marrow was removed for fixation. The tissue was then embedded in nitrocellulose, sectioned, and stained with hematoxylin-eosin-azure II.

## RESULTS

*Irradiation From External Sources.*—After x-irradiation the degenerative changes in the bone marrow were of similar type in the rabbit, mouse, rat, and chicken. With the LD 50/30 days, mitotic activity ceased and hematopoietic cells were destroyed, in every case erythroblasts before myelocytes. In the rabbit, as early as one-half hour after treatment, the erythroblasts were already reduced in number, and the remaining ones were predominantly late forms (normoblasts). Degeneration of hematopoietic cells continued at subsequent intervals, and by twenty-four hours erythroblasts had dropped to a minimum. Myelocytes, disappearing less rapidly and their destruction sometimes preceded by bizarre forms, reached a low point at 9 days. Megakaryocytes apparently suffered only occasional damage the first day, but by 2 days few were left. The debris was ingested by phagocytes, and the depleted marrow was replaced first by a gelatinous material and then by fat cells. Reticular cells were never found damaged. As the cells began to regenerate, there was an increase in mitotic figures, a preponderance of young forms of hematopoietic cells, and a diminution of fat cells.

In rabbits exposed to 400 r of x-rays, erythropoiesis in the bone marrow was again affected earlier than granulocytopenia, the results differing from those following the higher dose only in degree of damage and in time of its occurrence. Here fewer of the total number of cells were completely destroyed. Lobing of the maturing erythroblasts occurred abnormally early and was excessive. Megamyelocytes appeared, rivaling the megakaryocytes in size. These matured into giant granulocytes with 8 to 12 lobes to the nucleus and eventually degenerated in the marrow.

In rabbits exposed to 100 r of x-rays, the effects on the bone marrow were much less intense and less persistent than those which followed the 400 r dose. While mitosis stopped almost completely, it had been resumed by 3 hours after treatment and continued through the 14-hour interval at a level slightly above that seen in controls. At 8 hours a number of abnormal mitoses were observed; there were also a few dead cells, mostly erythroblasts, occasional nuclei with clumped chromatin, and rare megamyelocytes. From 14 hours through 21 days these marrows were no different from the controls.

In mice exposed to 350 r of x-rays, well below the LD 50/30 days for this species, the general progression of damage and repair of hematopoietic cells was like that observed in the rabbits, again with depletion first most marked in the erythrocytic series, but with myelocytes involved later. Recovery was indicated at 5 days, and was further advanced at 9 and 14 days.

In rats exposed to 600 r of x-rays, the LD 50/30 days, hematopoiesis, normally similar to that in mice, was almost completely eliminated by 2 days after treatment, the depleted areas filling with gelatinous or fatty marrow, but was nearly restored at the 31-day interval. At 3 hours erythroblasts were markedly reduced in number and there was considerable cellular debris. By 14 hours erythroblasts were almost completely gone. Myelocytopenia disappeared rather more slowly but by 2 days was practically absent. Regeneration, observed at 9 days with the return of erythropoiesis, was increased at 14 days with a preponderance of young forms. At subsequent intervals this function appeared normal, except for a possibly significant retrogression in the 21-day specimens. Myelocytopenia continued to be depressed until about 31 days.

The chicken bone marrow was affected by all doses from 25 to 1,000 r, the damage being roughly proportional to the size of the dose. Only 800 and 1,000 r resulted in the production of gelatinous marrow, and after these doses no permanent regeneration was observed in the 9- and 4-day duration of these experiments.

In 3-week chicks exposed to 800 r, erythropoiesis was completely removed at 1 hour and granulocytopenia was gone at 14 hours. Transient waves of regeneration were seen in the erythropoietic series at 2 to 14 hours and at 2 to 5 days; in the granulocytic series the recovery attempts were observed at 5 hours and again at 1 to 4 days. These recovery efforts were futile, however, and the marrow at 9 days was entirely aplastic. In the 11-week chickens two similar waves of regeneration were seen in the granulocytic series.

The presence of lymphatic nodules in the chicken marrow made it possible, in the same organ, to compare damage to lymphocytes with the effects on erythroblasts and myelocytes. Also, since erythropoiesis is intravascular and myelocytopenia is extravascular in the bone marrow of this species, it is less difficult to differentiate between very early forms of these two cell lineages than in the mammalian marrow where the two processes are intermingled.

Certain immature cells in the chick marrow proved more resistant to x-ray treatment than others. Most of the large lymphocytes of the lymphatic nodules

and the few stem cells among the myelocytes were intact at intervals when the small lymphocytes were extensively damaged and the basophil erythroblasts had been totally destroyed.

These differences in susceptibility among cell types were observed not only in the 3-week chicks given 800 r, but also in the 11-week chicks given 800 r and in the 3-week chicks given 400 r. In the older birds the small lymphocytes in the lymphatic nodules were dead at 1 and 3 hours, but the large lymphocytes were intact. All the basophil erythroblasts were dead in the sinuses, while extravascularly many normal myelocytes remained. Following 400 r in the younger chicks, the destruction was less severe but the differences in sensitivity were even clearer than after the higher dose. In the lymphatic tissue of the marrow, only some of the small lymphocytes were dead at  $\frac{1}{2}$  hour, and while the few hemocytoblasts present with the myelocytes appeared to be unaffected, all basophil erythroblasts inside the sinuses had been destroyed.

The pattern of degeneration and subsequent recovery of the bone marrow of rabbits exposed to fast neutrons was strikingly similar to that observed after x-irradiation. Three hours after exposure to 117 n\* the degeneration of erythroblasts was at its peak; 5 hours later the destruction was still prominent and quantities of debris were being phagocytosed. The damage to myelocytes was not conspicuous until 17 hours and reached a maximum at 26 hours, occurring later than the damage to erythroblasts, as in the x-irradiated animals. Progressive cellular depletion, the development of gelatinous marrow, and the eventual restoration of hematopoiesis followed.

In the bone marrow of mice exposed to fast neutrons, hematopoietic cells were destroyed to a degree related to the size of the dose. Especially after the lowest dose used, 65 n, it was quite clear that erythroblasts were the first of the hematopoietic cells destroyed.

Again, after exposure of mice to slow neutrons (400 arbitrary units), the same sequence of cellular destruction occurred. At the early intervals the bone marrow contained an increasing amount of debris and damaged hematopoietic cells, especially erythroblasts. By 12 hours myelocytes were fewer in number and groups of those present were pyknotic. The depletion increased up to 4 days, with gelatinous marrow as well as greatly widened sinuses replacing the cellular marrow. At the height of depletion the marrow was aplastic, except for spindle cells, plasma cells, some granulocytes, and, in the shaft, a few groups of normoblasts. By 7 days regeneration was well advanced. The sinuses were now only slightly dilated, and granulocytes were markedly increased throughout the marrow, especially in the shaft. Relatively few erythroblasts were present. Megakaryocytes were rare, except for an aggregation of them in one area just below the cartilage plate of one specimen. By 11 days there had been a great increase in hematopoiesis in the epiphysis and metaphysis, as well as in the shaft, with much more of it erythropoietic than before. At 2 weeks regeneration appeared to be complete.

\*The letter n signifies a purely arbitrary unit for measuring fast neutrons: 1 n is the amount of neutron radiation which produces a reading of 1 r on a Victoreen condenser-type r-meter equipped with a 100 r chamber. This meter is commonly used for measuring X and  $\gamma$  rays in r units.

*Irradiation From Internal Sources.*—When administered internally, certain radioactive isotopes, including some which are almost pure  $\beta$  emitters and others which emit primarily  $\alpha$  rays, affect bone marrow in much the same way, qualitatively, as does irradiation from external sources, especially x-rays and neutrons. Hematopoietic cells were destroyed, sinuses became dilated, and the depleted marrow became gelatinous. In contrast to the picture of sudden destruction and subsequent depletion of cells after x-irradiation, the depletion resulting from internal treatment occurred more gradually and was accompanied by very little cellular debris, as there was ample time for its removal by phagocytosis before it accumulated in large amounts. Also the effects on the marrow persisted, their duration depending on the rate of decay of the agent. The order of destruction of hematopoietic cells was the same here as after external irradiation, erythroblasts before myelocytes and myelocytes before megakaryocytes. Free macrophages, reticular cells, and fat cells were radioresistant. These differences between the several cell strains in sensitivity to irradiation were obvious in mice treated with strontium<sup>89</sup>, barium<sup>140</sup>-lanthanum<sup>140</sup>, phosphorus<sup>32</sup>, sodium<sup>24</sup>, plutonium, and radium, and in rats after strontium<sup>89</sup> and yttrium<sup>91</sup>. The location and type of "particle" emitted by the isotope modified the extent but not the quality of the injury. Thus, the short-ranged  $\alpha$  particles produced a relatively narrow rim of aplastic marrow around each trabecula in which they lodged. The much longer-ranged  $\beta$  particles produced much broader zones of damage; indeed, the marrow of the long bones of mice became completely aplastic after the deposition of fair amounts of the  $\beta$  emitting isotopes in bone.

#### CONCLUSIONS

In the bone marrow of mammals exposed to total body irradiation with x-rays and fast and slow neutrons, hematopoiesis is severely depressed, the degree of injury varying with the dose. The changes in the marrow cells after the injection of the radioactive isotopes, especially those that deposit in bone, are much the same as after the radiations coming from external sources. In all cases the erythropoietic cells are more susceptible to damage than the myelocytopoietic ones, and these in turn than the megakaryocytes.

Similar findings were demonstrated in chickens exposed to x-rays. In these animals the intravascular position of erythropoiesis makes the recognition of these cells easy even in hematoxylin and eosin preparations. The erythroblasts are at least as radiosensitive as the smaller lymphocytes of the nodules which are common in chicken marrow. The large lymphocytes are somewhat more resistant than the small ones.

Fat cells, free macrophages, and reticular cells (including those lining the sinuses) seem to be completely radioresistant to the dosage of irradiation we used.

These findings are in general agreement with the observations made by our group in comparing the relative radiosensitivity of the lymphatic versus myeloid cells of the spleen of rabbits and especially of mice and rats.

It is quite probable that those authors who described large numbers of degenerating lymphocytes in mammalian bone marrow after irradiation had confused these cells with degenerating erythroblasts. It may be that the extreme radiosensitivity of erythroblasts has often been overlooked because of the acute changes of the leucocytes in the peripheral blood, in contrast to the minor changes in the long-lived erythrocytes.

## REFERENCES

- Aubertin, C., and Beaujard, E.: Action des rayons X sur le sang et les organes hématopoïétiques, *Compt. rend. Soc. de biol.* 58: 217, 1905.
- Dunlap, C. E.: Effects of Radiation on the Blood and the Hematopoietic Tissues, Including the Spleen, the Thymus and the Lymph Nodes, in Shields Warren's *Effects of Radiation on Normal Tissues*, *Arch. Path.* 34: 562, 1942.
- Hagen, C. W., and Zirkle, R. E.: Unpublished work done under the Manhattan Project, 1946.
- Halberstaedter, L., and Simons, A.: Die Wirkung der Radium-, Katoden-, Röntgen- und Lichtstrahlen auf die blutbildenden Organe und das Blut, in Hirschfeld, H., and Hittmair, A., editors: *Handbuch der allgemeinen Hämatologie*, Berlin, 1933, Urban & Schwarzenberg, vol. I, part 2, p. 1419.
- Heineke, H.: Ueber die Einwirkung der Röntgenstrahlen auf Tiere, *München. med. Wchnschr.* 50: 2090, 1903.
- Heineke, H.: Experimentelle Einwirkung der Röntgenstrahlen auf das Knochenmark, über die Röntgentherapie der Leukämie und Pseudoleukämie, *tsche ztschr. f. Chir.* 78: 196, 1905.
- den Hoed, D., Levie, B., and Straub, M.: Serious Injury of the Blood in Consequence of Teleroentgen-Therapy of the Whole Body, *Acta radiol.* 19: 151, 1938.
- Jolly, J.: Sensibilité comparée des différents organes lymphoïdes aux rayons X, *Compt. rend. Soc. de biol.* 91: 354, 1924.
- Krause, P., and Ziegler, K.: Experimentelle Untersuchungen über die Einwirkung der Röntgenstrahlen auf tierische Gewebe, *Fortschr. a. d. Geb. d. Röntgenstrahlen* 10: 126, 1906.
- Lacassagne, A., and Lavedan, J.: Les modifications histologiques du sang consécutives aux irradiations expérimentales, *Paris méd.* 51: 97, 1924.
- Milchner, R., and Mosse, M.: Zur Frage der Behandlung der Blutkrankheiten mit Röntgenstrahlen, *Berl. klin. Wchnschr.* 41: 1267, 1904.
- Norris, W., Tompkins, P. C., and Finkle, R. D.: Unpublished work done under the Manhattan Project, 1946.
- Selling, L., and Osgood, E. E.: The Action of Benzol, Roentgen Rays and Radioactive Substances on the Blood and Blood-Forming Tissues, in Downey, Hal, editor: *Handbook of Hematology*, New York, 1938, Paul B. Hoeber, Inc., vol. 4, p. 2689.
- Snyder, R., and Finkle, R. D.: Unpublished work done under the Manhattan Project, 1946.
- Zirkle, R. E., and Raper, J. R.: Unpublished work done under the Manhattan Project, 1946.

# THE STRUCTURE OF THE GIANT CELLS IN THE BLOOD-FORMING ORGANS

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## INTRODUCTION

**N**ORMALLY the blood-forming organs, particularly the bone marrow, contain a few scattered giant cells or exceedingly large cells. The megakaryocytes, osteoclasts, and possibly the multinucleated plasma cells belong to this group and play a role in the physiologic processes of the various blood-forming organs in which they are found. More numerous and extremely variegated in structure are the giant atypical mononuclear cells and the polymorphonuclear and multinucleated giant cells frequently observed concomitant with or partaking in pathologic processes in the blood-forming organs. It is the purpose of this paper to make readily accessible brief morphologic descriptions and accompanying photomicrographic representations of both typical and atypical giant cells, much in the manner of an atlas in miniature.

The material and records available at the Army Institute of Pathology and its associated Lymphatic Tumor Registry are admirably suited to this purpose, comprising as they do numerous examples of all the common pathologic processes and most of the rarer ones. Furthermore, the diagnostic identification of the more controversial processes is usually a composite of the studies and opinions of many different pathologists representing diverse schools of thought and including specialists in the fields of hematology and hematopathology as well as general pathology. For example, only those cases were utilized in studying the giant cells in reticulum cell sarcoma of the lymph nodes in which the diagnosis of reticulum cell sarcoma was essentially agreed upon by all the consultants.

In the preparation of this paper the author has made frequent use of Haythorn's<sup>1</sup> exhaustive review of the literature on the giant cells with especial reference to the foreign body group.

## MATERIALS AND METHODS

The material available comprised clinical records, autopsy or surgical protocols, and formalin-fixed tissues, with the addition, in many cases, of dry-fixed smears or impressions of the hematopoietic organs. All cases had been studied in hospitals. Paraffin sections were prepared from all formalin-fixed tissues and were stained with hematoxylin and eosin. In instances of disturbances in the metabolism of lipids, various fat stains were used. To meet the requirements of individual cases, Wilder's method for reticulum, MacCallum's stain for bacteria, and Kinyoun's stain for acid-fast bacilli were employed. The dry-fixed smears and impressions were stained by the Romanowsky methods, Wright's stain or, preferably, Wright-Giemsa or May-Grünwald-Giemsa, as employed by Slider and Downey<sup>2</sup> and Kolouch.<sup>3</sup> Impressions are obtained by touching a fresh cut surface of tissue to a clean slide, so lightly that but a single

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layer of cells adheres to the glass. The slide is immediately air-dried without the application of heat and is then ready for staining. The imprint method is preferable to smears because the cells are less likely to be distorted and ruptured in the process.

The photomicrographs were all taken at the same magnification ( $\times 2000$ ) so that ready comparison of the size of the various structures could be made by the reader. Account must be taken, however, of the shrinkage due to fixation when comparing the size of cells in sections and imprints. The higher power was employed because a number of the cells herein discussed have been depicted at extremely low magnification or not at all in the literature, although the majority have been excellently portrayed.

#### OBSERVATIONS

A. THE MEGAKARYOCYTES.—In 1890 Howell<sup>4</sup> differentiated a new type of giant cell in the bone marrow from the osteoclast type originally described by Kölliker.<sup>5</sup> To this separate class of cells Howell gave the name "megakaryocyte." In 1906 Wright<sup>6</sup> traced the origin of the blood platelets from Howell's megakaryocytes. The morphologic descriptions which follow will serve as a basis for comparison of these cells with the various more or less characteristic giant cells to be found in Hodgkin's disease and reticulum cell sarcoma, structures with which megakaryocytes are closely identified by some observers. For further details of megakaryocytic morphology the reader is referred to the recent reviews by Rosenthal<sup>16</sup> and by Dameshek and Miller.<sup>7</sup>

##### 1. Megakaryoblast.—

*Morphologic Characteristics in Dry-Fixed Smears or Imprints:* This cell is slightly larger than the myeloblast (hemocytoblast) which it rather closely resembles. The megakaryoblast has a round or slightly irregular nucleus and a thin nuclear membrane. The nuclear pattern is produced by small chromatin particles arranged in a fine sievelike or a dark regularly stippled design, somewhat coarser than that of the myeloblast. The parachromatin spaces (karyoplasm) are characteristically pink and sharply demarcated from the chromatin particles, but the nuclear pattern is generally slightly less leptochromatic than that of the myeloblast. One or more nucleoli may be present. The nuclear-cytoplasmic ratio favors the nucleus. The cytoplasm, which forms a narrow rim about the nucleus, is basophilic and nongranular. I have not included an illustration of this cell, which actually is not a giant cell; however, the papers of Jürgens and Graupner,<sup>8</sup> Strumia,<sup>9</sup> Rohr,<sup>10</sup> and Dameshek and Miller<sup>7</sup> present good colored representations of this cell type.

*Morphologic Characteristics in Tissue Sections:* The megakaryoblast is slightly larger than the hemocytoblast (Fig. 1). Its cytoplasm shows a lessening in basophilic properties. The nuclear membrane is thicker than that of the hemocytoblast and the chromatin masses are coarser, although a nucleolus occasionally may be made out. This cell can be identified in sections only by the nature of its associates in areas of intense megakaryocytic metaplasia.

*Origin:* Ordinarily this cell is derived from the myeloblast (hemocytoblast) (Ferrata and Negreiros-Rinaldi<sup>11</sup> 1915, Downey and Nordland<sup>12</sup> 1939) and from



the reticulum cell (hemohistioblast) (Custer<sup>13</sup> 1933, Downey and Nordland<sup>12</sup> 1939, and Dameshek and Miller<sup>7</sup>).

## 2. Promegakaryocyte.—

*Characteristics in Dry-Fixed Smears or Imprints:* The size of this cell varies from that of the megakaryoblast to well over 20 microns in diameter (Figs. 2 and 3). The nuclear membrane is thin; the nucleus is larger than that of the megakaryoblast; and the nuclear pattern may retain the fine chromatin-parachromatin features of the latter, although it is usual for masses of clumped chromatin to make their appearance in a few areas at this stage. The clumped chromatin is presented in Fig. 3, whereas the cell in Fig. 4 has largely retained the megakaryoblastic pattern. The nucleus may be round or nuclear lobulation may begin at this stage. In general, the nuclear texture is heavier and nucleoli are usually absent. The cytoplasm is basophilic, scant or abundant, and often presents a fine azurophil granulation about the nucleus. Frequently, as seen in the excellent figures of Downey and Nordland<sup>12</sup> and Dameshek and Miller,<sup>7</sup> granular, occasionally nongranular, platelet-like forms are to be found at the cytoplasmic periphery. The larger cells of this group (Figs. 4, 5) are actually intermediate between promegakaryocytes and megakaryocytes.

*Characteristics in Tissue Sections:* In tissue sections the promegakaryocyte is larger than the megakaryoblast; both nucleus and cytoplasm are increased in size, and the nuclear membrane is thicker. The chromatin masses are larger and coarser, and the vesicular appearance of the hemoctyblast is entirely lost. Lobulation of the nucleus may occur (Fig. 6) or the nucleus may remain round but slightly irregular (Fig. 7). If an appropriate fixative, formol-corrosive (Downey<sup>14</sup>), and stain, Giemsa, Wright's or Dominici's (Slider and Downey<sup>2</sup>), are employed a small azurophilic or acidophilic granular area appears in the cytoplasm eccentrically near the nucleus and gradually spreads until the entire cytoplasm becomes granular. Unfortunately, ordinary tissue methods fail to bring out cytoplasmic detail in cells of this series.

*Origin:* Ordinarily the promegakaryocyte evolves from the megakaryoblast although in intense reticular stimulation it can also be derived from the reticulum cell.

## 3. Megakaryocyte.—

*Characteristics in Dry-Fixed Preparations:* The megakaryocyte is usually a very large cell, well over 40 microns in diameter. The nuclear membrane is thick, and the nucleus is large, polymorphous (Fig. 8), and extremely complicated in form. The original chromatin-parachromatin distinction may be all but obscured by the coarsening of the chromatin masses, although at times distinct areas of parachromatin can be observed (Fig. 5). The cytoplasm is abundant, basophilic, and contains numerous azurophil granules. Gradually the azurophil granules form into groups surrounded by clearer areas of cytoplasm. Pseudopodial processes at the periphery of the cell are frequently found to contain these platelet-like formations. For further cytologic details and colored figures of these cells the reader is referred to the works of Rohr<sup>10</sup> and Dameshek and Miller.<sup>7</sup>

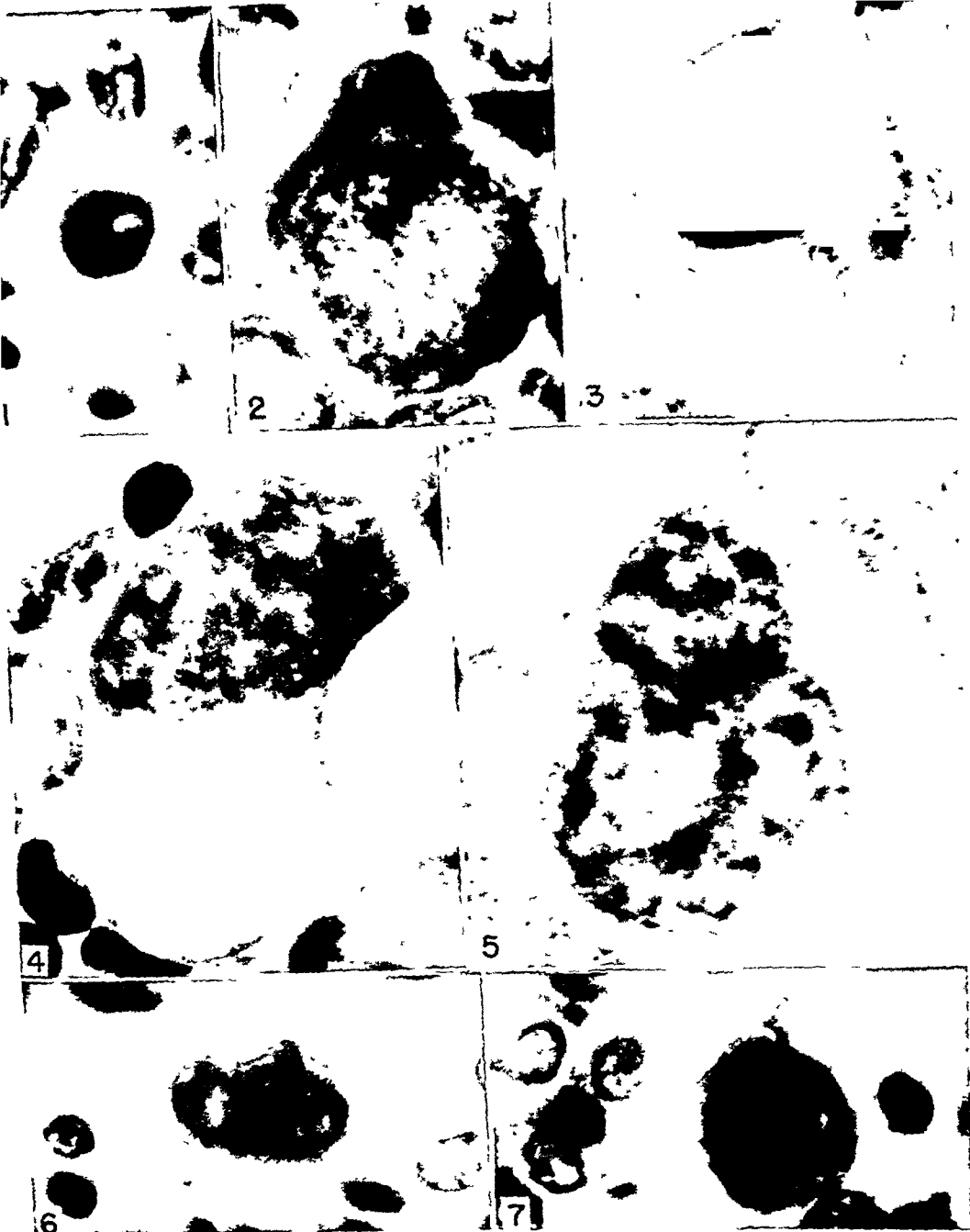


PLATE I

Fig. 1.—Megakaryoblast, section of spleen in myeloid metaplasia. Hematoxylin and eosin stain, X2000. AIP Neg. 97013

Fig. 2.—Early promegakaryocyte, sternal bone marrow aspirate. May-Grünwald-Giemsa stain, X2000. AIP Neg. 96993

Fig. 3.—Promegakaryocyte, sternal bone marrow aspirate. Wright's stain; X2000. AIP Neg. 97006.

Fig. 4.—Promegakaryocyte, sternal bone marrow aspirate. May-Grünwald-Giemsa stain; X2000. AIP Neg. 96992.

Fig. 5.—Intermediate megakaryocyte, sternal bone marrow aspirate. Wright's stain; X2000. AIP Neg. 97005.

Fig. 6.—Promegakaryocyte, section of spleen in myeloid metaplasia. Hematoxylin and eosin stain; X2000. AIP Neg. 97015.

Fig. 7.—Promegakaryocyte, section of spleen in myeloid metaplasia. Hematoxylin and eosin stain; X2000. AIP Neg. 97014.

*Characteristics in Tissue Sections:* In sections the megakaryocyte may have a diameter as great as 40 microns. The cell outline may be roughly spherical or irregular. The nucleus is large and polymorphous (Fig. 9), the nuclear membrane thick. The chromatin network is composed of coarse dense chromatin strands. Nucleoli are present but usually indistinct. The cytoplasm is abundant and slightly acidophilic. With appropriate fixation and staining, diffuse or grouped azurophil granules are found throughout the cytoplasm. The periphery of the cell is frequently provided with irregular, fingerlike processes of various shapes. In the bone marrow these pseudopodia may be found projecting into the sinuses (Downey,<sup>14</sup> his Fig. 17). Platelets are formed by the fragmentation of pseudopodial cytoplasm, the azurophil granules serving as the chromomere and the fragments of cytoplasm as the hyalomere of the blood platelet.

*Origin:* Usually the megakaryocyte is derived from the promegakaryocyte, rarely from the polykaryocyte (Di Guglielmo<sup>15</sup> and Rosenthal<sup>16</sup>).

*Function:* These cells serve to form the blood platelets (Wright,<sup>6</sup> Bunting,<sup>17</sup> and Downey<sup>14</sup>).

*4. Dividing Forms of Megakaryocytes.*—The cells of the megakaryocytic series reach maturity by means of multipolar mitoses (Rohr<sup>10</sup> and Maximow and Bloom<sup>18</sup>), in which the cytoplasm undergoes no constriction and the daughter nuclei fuse at once into an increasingly pachychromatic, polymorphous nucleus. The fully mature megakaryocyte probably does not undergo further cell division.

*5. Polykaryocyte.*—Di Guglielmo<sup>15</sup> introduced the concept of the fusion of several histioid elements into a syncytium with multiple nuclei and but a single cell body, which in turn could serve as a source of megakaryocytes. Rosenthal<sup>16</sup> (his Figs. 11 to 13) and Dameshek and Miller<sup>7</sup> (their Fig. 5) depict such cells. The latter authors believe that these cells are probably identical with osteoclasts. Depicted in our Fig. 10 is a multinucleated megakaryocyte which possesses three discrete nuclear masses yet retains the cytoplasmic and nuclear detail of an ordinary megakaryocyte (compare this cell with Figs. 5 and 8). The concept of a polykaryocytic origin of the megakaryocyte has not received general acceptance. Fig. 10 also should be compared with Fig. 47, an osteoclast obtained from a dry-fixed preparation of the bone marrow. The osteoclast, in contrast to the multinucleated megakaryocyte, presents a marked reticulum cell or histiocytic type of nucleus and deeply basophilic cytoplasm lacking azurophil granules.

*6. Degenerated Megakaryocytic Forms Encountered in the Normal Bone Marrow.*—Upon completion of its platelet-forming function, the cytoplasm of the megakaryocyte, which now has become greatly depleted, disintegrates (Fig. 11). The polymorphous nucleus becomes dark-staining, shrunken, pyknotic, and stripped of almost all of its cytoplasm (Fig. 12). Degenerated megakaryocytic forms are readily confused with the degenerated forms of Reed-Sternberg cells found in Hodgkin's disease (Figs. 26 and 27); therefore, it is imperative that the differential study of these cell types be made before degeneration has progressed to any extent.



# PLATE II

Fig. 8—Adult megakaryocyte, sternal bone marrow aspirate. May-Grunwald-Giemsa stain, X2000. AIP Neg. 97614.

Fig. 9—Adult megakaryocyte, section of spleen in myeloid metaplasia. Hematoxylin and eosin stain, X2000. AIP Neg. 97243.

Fig. 10—Multinucleated megakaryocyte; sternal bone marrow aspirate. May-Grunwald-Giemsa stain, X2000. AIP Neg. 96991.

Fig. 11—Degenerating megakaryocyte, section of spleen in acute aplastic anemia. Hematoxylin and eosin stain, X2000. AIP Neg. 97016.

Fig. 12—Pknotic megakaryocyte nucleus, section of bone marrow in idiopathic pulmonary fibrosis. Hematoxylin and eosin stain, X2000. AIP Neg. 97254.

### 7. Pathologic Megakaryocytic Forms.—

a. In idiopathic (essential) thrombocytopenic purpura the megakaryocytes have been described in detail in the works of Frank,<sup>10</sup> Limarzi and Schleicher,<sup>20</sup> and Dameshek and Miller.<sup>7</sup> In brief, the number of these cells in the bone marrow is normal or increased; many of the adult megakaryocytes present absence of azurophil granulation or decrease in pseudopodial and platelet formation (Fig. 13). The promegakaryocytes show an abortive pseudopodial formation which results in the production of giant, atypical, nongranular platelets. There may be a decrease in the number of megakaryoblasts and an increase in degenerated forms. The cytoplasm of the latter may show vacuolization and hyalinization, and the nuclei hyperlobulation, in addition to the features of degenerated forms already described.

b. In hemorrhagic or hemolytic anemias megakaryocytes are increased but platelet formation is abundant.

c. In hemophilia, polycythemia vera, secondary polycythemia, Gaucher's disease, Felty's syndrome, and aplastic anemia, to mention but a few conditions, quantitative but no qualitative changes in megakaryocytes have been described.

d. In myeloid megakaryocytic hepatosplenomegaly cells in all stages of megakaryocytic development are found in the peripheral blood along with bizarre atypical hypergranular or nongranular platelets. Even the megakaryoblasts and promegakaryocytes present atypical abortive pseudopodial platelet formation, spleen, liver, and lymph nodes may show myeloid metaplasia of a megakaryocytic type, but the marrow is not leukemic and may even possess fewer than the normal number of megakaryocytes (Downey<sup>12</sup>). Paseyro<sup>21</sup> (his Figs. 119 and 122) depicts megakaryocytes in dry-fixed preparations from the spleens of patients with this disease.

e. In chronic myelogenous leukemia any of the conditions existing in myeloid megakaryocytic hepatosplenomegaly may be found, with the addition of similar changes in the megakaryocytic series in the bone marrow and other areas of leukemic "infiltration."

f. In pernicious anemia, Rohr<sup>10</sup> (his Fig. 47) and Paseyro<sup>21</sup> (his Fig. 45) depict marked hypersegmentation of the megakaryocytic nucleus comparable to the hypersegmentation of the neutrophilic leukocytes in the bone marrow in this disease. Jones<sup>22</sup> (his Figs. 10 and 11 and Plate II) found megakaryocytes

### Plate III

Fig. 13.—Mature megakaryocyte lacking pseudopodial platelet formation. Sternal bone marrow aspirate. May-Grünwald-Giemsa stain;  $\times 2000$ . AIP Neg. 98047.

Fig. 14.—Multinucleated reticular promegaloblasts. Sternal bone marrow aspirate in pernicious anemia. Dr. R. P. Custer's case. May-Grünwald-Giemsa stain;  $\times 2000$ . AIP Neg. 97885.

Fig. 15.—Three stages of megakaryocytic development. Section of bone marrow in megakaryocytic leukemia. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97606.

Fig. 16.—Mature megakaryocyte. Section of lymph node in megakaryocytic leukemia. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97897.

Fig. 17.—"Leukemic" megakaryocyte. Section of spleen in megakaryocytic leukemia. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97899.

Fig. 18.—"Leukemic" megakaryocyte. Section of bone marrow in megakaryocytic leukemia. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97604.

Fig. 19.—"Leukemic" megakaryocyte. Section of spleen in megakaryocytic leukemia. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97896.

Fig. 20.—Atypical mitotic figure in megakaryocyte. Section of spleen in megakaryocytic leukemia. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97603.



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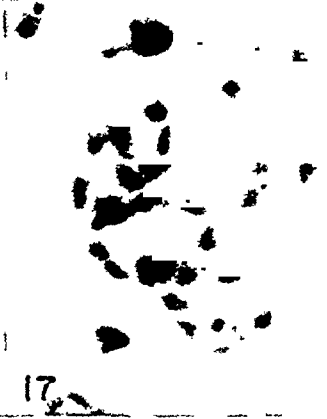
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PLATE III  
(For legend, see opposite page)

with "extremely basophilic cytoplasm and mottled areas of hyaloplasm without evidence of platelet formation" in the bone marrow of patients with pernicious anemia in relapse. Paceyro<sup>21</sup> (his Figs. 45 and 46) has noted an intense megakaryocytic reaction in one of his patients with pernicious anemia. It should be noted that in this disease another type of multinucleated giant cell occasionally is found in the bone marrow (our Fig. 14); it is made up of reticular promegaloblasts with multiple discrete nuclei in a single cytoplasmic mass, which stains with the same intense basophilia as does the cytoplasm of individual promegaloblasts. Although the cell illustrated is binucleate, others containing as many as nine nuclei were observed in the same marrow by Dr. R. P. Custer. Rohr<sup>10</sup> (his Fig. 44) illustrates similar reticular-promegaloblastic cell masses in pernicious anemia.

g. In secondary toxic thrombocytopenia, Rohr<sup>10</sup> (his Fig. 102) depicts abnormal and incomplete platelet formation in restricted portions of the megakaryocytic cytoplasm.

h. In megakaryocytic leukemia it is possible to observe what may be termed a "malignant" megakaryocytic series. With the leukemic processes centered in cells of the developing megakaryocytic type, one can compare the structure of the resultant leukemic megakaryocytes on the one hand with definitive megakaryocytes and on the other hand with Reed-Sternberg cells of Hodgkin's disease. The leukemic megakaryocytes arise partly from the hemocytoblasts and partly from the reticulum cells (mesenchymal cells) in the bone marrow, lymph nodes, spleen, and other sites of leukemic "infiltration." Three stages in the early development of these cells in the bone marrow are depicted in our Fig. 15. The structure of the young forms in tissue sections is similar to that already described for cells of the definitive megakaryocytic series. The more fully developed forms, such as the cell from a lymph node in Fig. 16, may likewise closely resemble the definitive megakaryocyte (compare with Fig. 9), although many of them present marked variations. Their nuclei may undergo extreme hyperlobulation (Fig. 17) without degenerative changes, the individual nucleus frequently being massive (Figs. 18 and 19). In spite of these changes it should be noted that the nuclear structure remains vesicular, and if the occasional clumps of coarse chromatin are present, they cling to a moderately thick nuclear membrane (Figs. 17 and 19). Two or three of the giant lobes may contain large abnormal

#### Plate IV

Fig. 21.—Atypical enlarged reticulum cell. Section of lymph node in Hodgkin's disease treated with nitrogen mustard. Hematoxylin and eosin stain; X2000. AIP Neg. 97002.

Fig. 22.—Tripolar mitosis in Reed-Sternberg cell and atypical enlarged reticulum cell. Section of lymph node in Hodgkin's disease treated with nitrogen mustard. Hematoxylin and eosin stain; X2000. AIP Neg. 97000.

Fig. 23.—Atypical binucleated enlarged reticulum cell. Section of lymph node in Hodgkin's disease treated with nitrogen mustard. Hematoxylin and eosin stain; X2000. AIP Neg. 97003.

Fig. 24.—Small Reed-Sternberg cell. Section of lymph node in Hodgkin's disease. Hematoxylin and eosin stain; X2000. AIP Neg. 98052.

Fig. 25.—Large Reed-Sternberg cell. Section of lymph node in Hodgkin's disease treated with nitrogen mustard. Hematoxylin and eosin stain; X2000. AIP Neg. 97258.

Fig. 26.—Degenerated Reed-Sternberg cell. Section of lymph node in Hodgkin's disease treated with nitrogen mustard. Hematoxylin and eosin stain; X2000. AIP Neg. 97258.

Fig. 27.—Degenerated Reed-Sternberg cell. Section of lymph node in Hodgkin's disease treated with nitrogen mustard. Hematoxylin and eosin stain; X2000. AIP Neg. 97257.

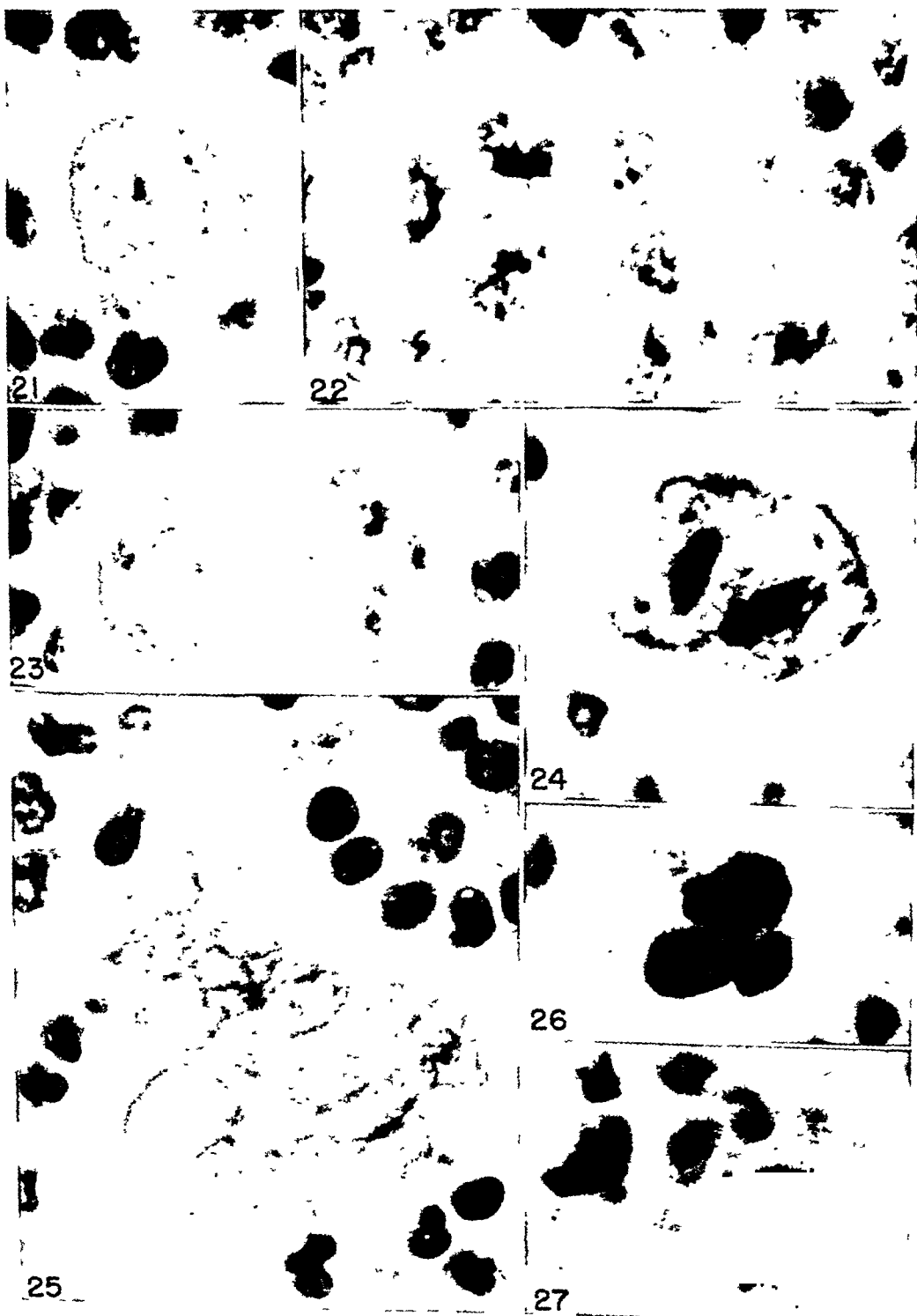


PLATE IV

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basophilic nucleoli; however, they are not a conspicuous feature in the majority of leukemic megakaryocytes, although they are more prominent than in definitive megakaryocytes. Giant bizarre nuclei of the type depicted in Figs. 18 and 19 are not found in definitive megakaryocytic series (compare with Fig. 9). On the other hand, in Reed-Sternberg cells the acidophilic nucleoli are a prominent feature, and cells corresponding to those of the definitive megakaryocytic series (for instance the cell in Fig. 16) are not found in company with the Reed-Sternberg cells in extramedullary malignant lymphogranulomatous areas. The mitoses, as is to be expected in the leukemic megakaryocytic group, are extremely bizarre (Fig. 20). For additional information on cytologic detail it is imperative that such cells be studied by the dry-fixed smear or imprint technique as well as in tissue sections.

**B. GIANT CELLS IN HODGKIN'S DISEASE.**—The characteristic giant cells in Hodgkin's disease were described first by Sternberg<sup>23</sup> and Reed.<sup>24</sup> Reference to their careful and complete descriptions of these cells as they appear in tissue sections should go far in removing the difficulties encountered in the identification of giant cells of this type. For this reason I shall quote their original descriptions.

Sternberg,<sup>23</sup> p. 23 (1898) writes: “. . . ferner grössere, protoplasmareiche Zellen mit grossen, dunkelgefärbten Kernen, die nicht so selten mehrfach zu zweien und dreien oder auch gelappt erscheinen. Vereinzelt solche Zellen besitzen eine grössere Anzahl (bis zu fünf und sechs) Kernen. An manchen sind die Kerne auffallend gross, rund und enthalten sich mit Eosin tingierende Kernkörperchen, oder, was seltener der Fall ist, die Kerne sind blass gefärbt und enthalten einen oder zwei sich mit Eosin farbende Nucleolen. In der Mehrzahl sind sie überchromatinreich. Diese Zellen liegen zumeist frei zwischen den Zügen lockeren Bindegewebes, auch denselben an und manchmal so innig und sind auch mit plumpen Ausläufern versehen, dass sie mit dem Stroma in Zusammenhang zu sein scheinen.”

His Figs. 1 to 4 illustrate the cells he describes.

In 1902 Reed<sup>24</sup> describes these cells in the following manner:

“The nucleus is always large in proportion to the size of the cell. It may be single or multiple. If single, it is usually round. Bean-shaped and irregularly indented nuclei are common. If multiple, the nuclei may be arranged peripherally in the cell or heaped in the center. Eight or ten nuclei have been seen in a single cell. The chromatin network is prominent in these nuclei, and one or more large nucleoli are always present. The nucleoli are usually oval but they may be of any shape. The nucleoli always take a contrasting stain in the double stains; they have an affinity for acid dyes. No definite mitotic figures were ever seen in these cells. Direct division was frequently observed. The protoplasm is usually homogeneous and stains well. It may appear granular, show vacuolization, or contain fat or pigment granules. Cells having bizarre and irregular nuclei are found in the oldest growths. These giant cells, so far as our observation reaches, are peculiar to this growth and are of great assistance in diagnosis.”

Her Figs. 2 to 5 illustrate these cell types.

Shown in our Fig. 21 is the enlarged reticulum cell in a case of Hodgkin's disease and in Fig. 22 a similar cell with two large acidophilic nucleoli, thin nuclear membrane, relatively few particles of chromatin, and scant faintly acidophilic cytoplasm. A very similar but binucleate cell of this type is found in

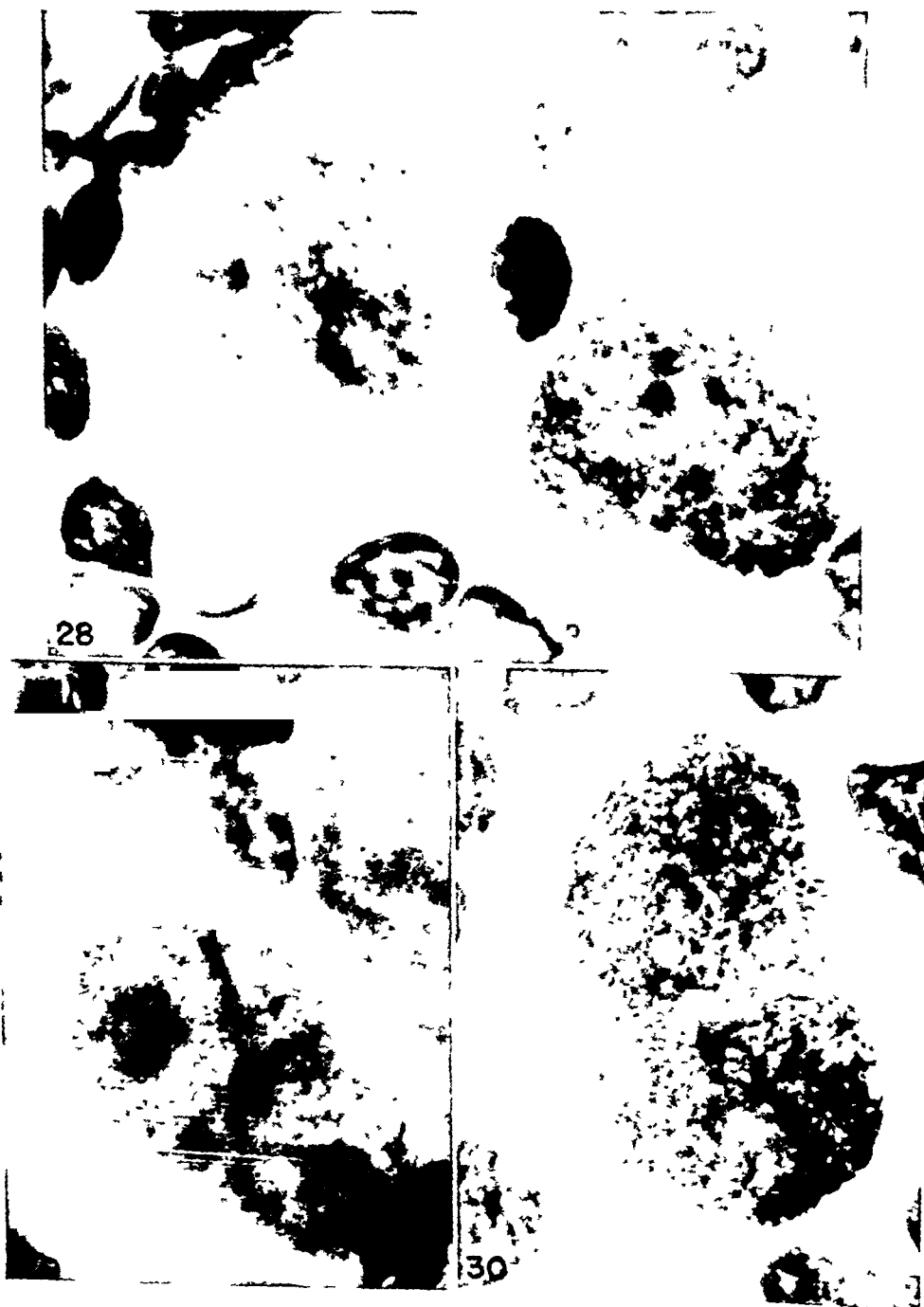
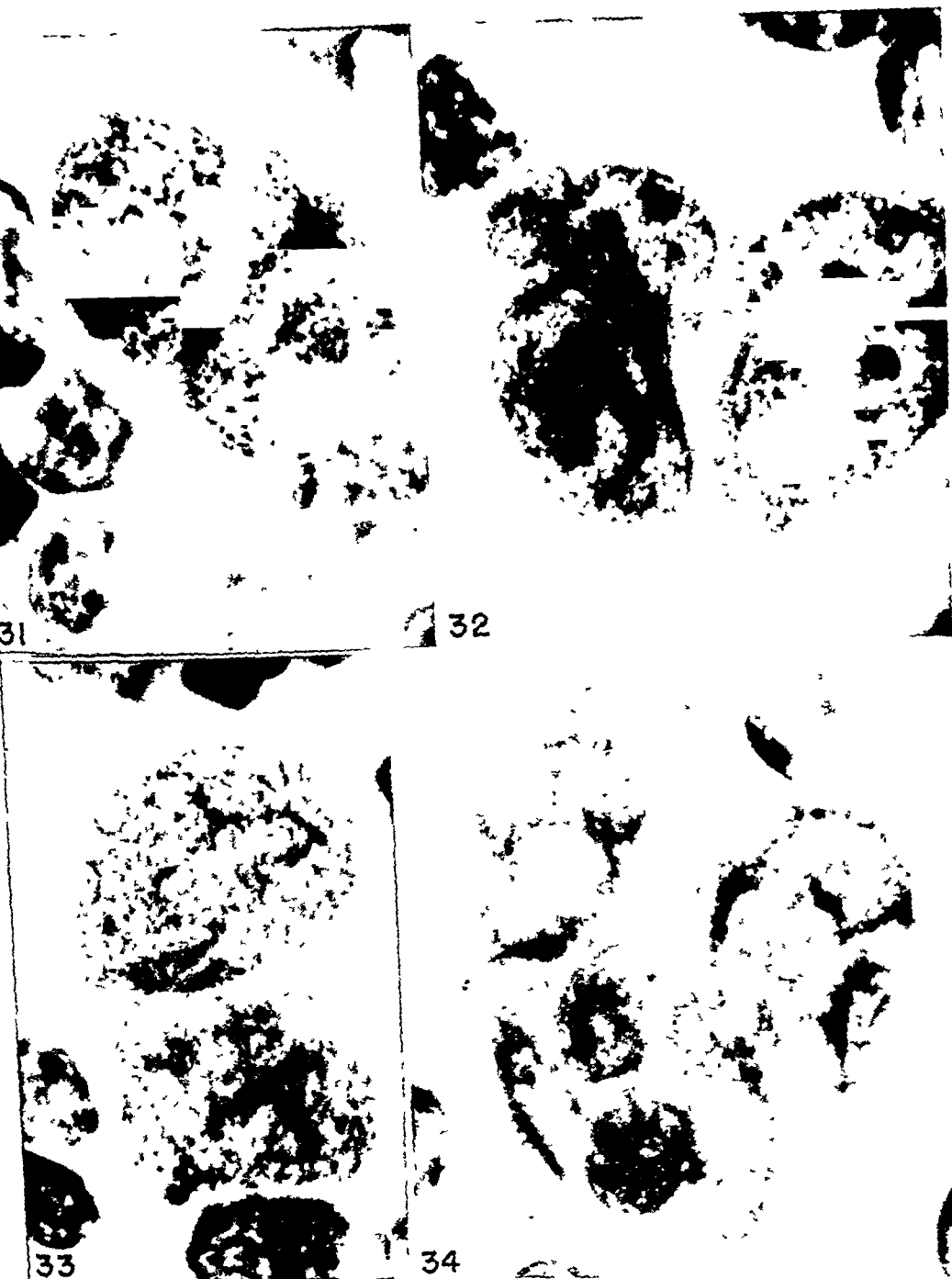


PLATE V

- Fig 28—Two atypical enlarged reticulum cells. Imprint of lymph node in Hodgkin's disease. Wright-Giemsa stain, X2000. AIP Neg 97902.
- Fig 29—Atypical enlarged reticulum cell. Imprint of lymph node in Hodgkin's disease. Wright-Giemsa stain, X2000. AIP Neg. 98049.
- Fig 30—Atypical binucleated enlarged reticulum cell. Imprint of lymph node in Hodgkin's disease. Wright-Giemsa stain, X2000. AIP Neg 97903.

Fig. 23. These cells should be compared with Figs. 1 and 7, developing megakaryocytes in tissue sections, which lack the large acidophilic nucleoli and scant chromatin pattern of Reed-Sternberg cells. The simpler form of giant lobulated Reed-Sternberg cell is depicted in Fig. 24. The large round or rod-shaped acidophilic nucleoli, so prominent in these cells, and their relatively moderate chromatin content are in sharp contrast to the nuclear characteristics of intermediate and adult megakaryocytes as seen in Figs. 6, 7, and 9. If further differentiating criteria are required, special fixation and staining (Downey<sup>14</sup>) will reveal the azurophil granulation and granular pseudopodial platelet formation on the part of megakaryocytic cytoplasm in tissue sections, two characteristics which are not features of the cytoplasm of Reed-Sternberg cells. Shown in Fig. 25 is one of the larger bizarre cells of the Reed-Sternberg type with excessive nuclear lobulation. Degenerated forms of Reed-Sternberg cells are a constant feature throughout the course of Hodgkin's disease, and they become even more apparent after the application of the various forms of therapy. Such cells undergoing pyknosis or lysis are shown in Figs. 26 and 27. It is not surprising, when we compare these cells with the degenerated forms of megakaryocytes (Figs. 11 and 12) and note the over-all resemblance between the pyknotic polymorphonuclear and multinucleated forms, that these two entire groups are occasionally identified as one, especially since degeneration may occur at any stage of development of the Reed-Sternberg cell. The observation of Reed<sup>23</sup> that mitoses are never found in these cells has been refuted by more recent findings of multipolar mitoses, as illustrated in Fig. 22.

In the dry-fixed impressions of the blood-forming organs, stained by the May-Grünwald-Giemsa technique, the disparity in structure between megakaryocytes and Reed-Sternberg cells is readily apparent. Paseyro<sup>21</sup> has a comprehensive series of photomicrographs of such preparations (his Figs. 69 to 73 and 75 to 78). In our Fig. 28 is shown the enlarged atypical reticulum cell of this disease. It is comparable to the cell of Fig. 22 as seen in tissue sections. The large size and beginning lobulation of the nucleus become more apparent. The chromatin is arranged in small angular pieces irregularly distributed throughout the nucleus, so as to leave distinct colorless parachromatin spaces. The nucleoli are as yet small, round or irregular, and deeply basophilic. The cytoplasm is abundant and consists of a large amount of flaky, granular, basophilic spongioplasm against a colorless background of hyaloplasm. Very rarely a few scattered azurophil granules are to be found within the cytoplasm, but in only an occasional cell, and the granules are few and never show a tendency to group or clump. Presented in Fig. 29 is a similar giant mononuclear cell with several prominent basophilic nucleoli. Depicted in Fig. 30 is a binucleate cell of the Reed-Sternberg type, lacking prominent nucleoli. Comparison of this illustration with those of the developing megakaryocytes (Figs. 2 and 3), in which the same technique was utilized, reveals a chromatin-parachromatin distinction of the nuclear patterns of the two types but increased clumping of the chromatin particles, absence of numerous or prominent basophilic nucleoli, and beginning azurophil granulation in the cytoplasm about the nucleus of cells of the megakaryocytic series.



## PLATE VI

Fig 31—Small Reed-Sternberg cell Imprint of lymph node in Hodgkin's disease Wright-Giemsa stain, X2000 AIP Neg 98050

Fig 32—Reed-Sternberg cell Imprint of lymph node in Hodgkin's disease Wright-Giemsa stain, X2000 AIP Neg 97901

Fig 33—Small Reed-Sternberg cell Imprint of lymph node in Hodgkin's disease Wright-Giemsa stain, X2000 AIP Neg 98048

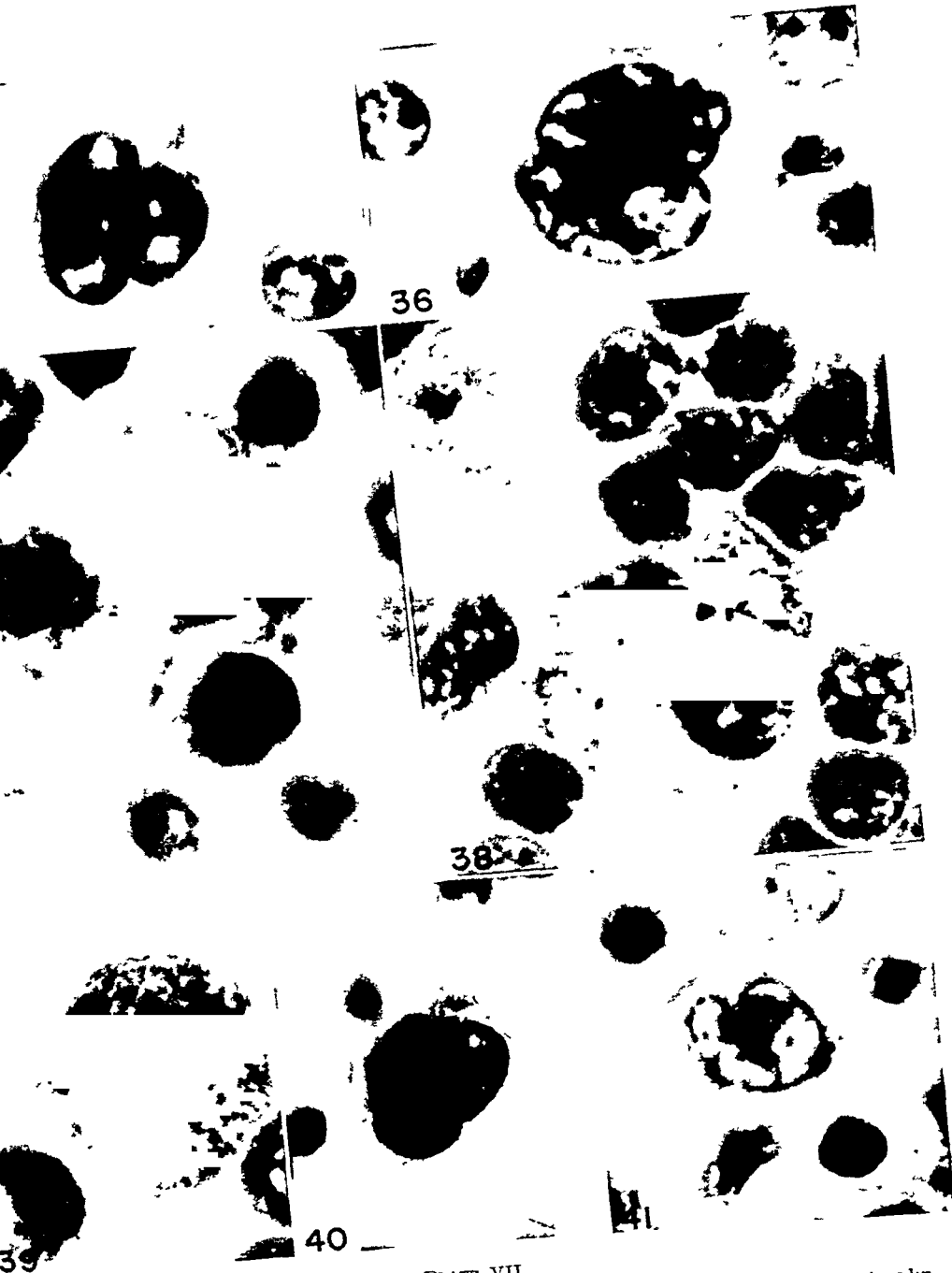
Fig 34—A part of a large Reed-Sternberg cell Imprint of lymph node in Hodgkin's disease Wright-Giemsa stain, X2000 AIP Neg 97905

In Figs. 31 to 34 is revealed lobulation of the nuclei of the Reed-Stern cells, increasing until they become giant, bizarre, and polymorphous. It is to be noted that the fine reticular chromatin network with its chromatin-para-chromatin distinction is retained until and beyond the stage of actual beginning cellular degeneration. The nucleoli, which may reach diameters up to 7 microns (Fig. 34), retain their deep, clear, basophilic staining properties. Instead of containing one or two large nucleoli, each nuclear lobe may be spotted with from six to eight small basophilic nucleoli (Fig. 33). The cytoplasm remains as described in the earlier developmental stages. The fully developed Reed-Sternberg cells should be compared with Figs. 5 and 8, with the more mature megakaryocytes, or with the corresponding figures of adult megakaryocytes from the cases of Dameshek and Miller.<sup>7</sup> For the more mature megakaryocytes natural pyknosis has gone on to produce a pachychromatic nuclear pattern; large or numerous basophilic nucleoli are absent or masked, and clumping of azurophil granulation, if not pseudopodial platelet formation, is usually apparent in the cytoplasm. Thus, the value of dry-fixed, imprint preparations is enhanced in cases in which Hodgkin's disease of the blood-forming organs is to be differentiated from those primary or secondary affections of the same organs in which megakaryocytic hyperplasia, "infiltration," or metaplasia is a prominent feature.

*Function:* The enlarged, abnormal reticulum cells of Hodgkin's disease are rarely found to be phagocytic, according to Jaffe.<sup>25</sup> But at the Army Institute of Pathology we have observed a case in which the Reed-Sternberg cells contained nuclear remnants and others, lipids. In the latter instance cells simulating Touton giant cells were observed, although the central ring of nuclei presented the nuclear pattern typical of Reed-Sternberg cells rather than of histiocytes.

It should also be emphasized that even the earlier authors mentioned non-specific foreign body giant cells in the primary dyscrasias of the blood-forming organs, in addition to the various specific giant cell forms. Thus, in Hodgkin's disease, Reed<sup>24</sup> describes a "second form" of giant cell, similar to the Langhans' giant cell and Mallory<sup>26</sup> foreign body giant cells. It is interesting that Langhans<sup>27</sup> himself noted the occurrence of cells identical to those which he had observed in tuberculosis, in cases of what he then termed "malignant lympho-sarcoma."

C. GIANT CELLS OF RETICULUM CELL SARCOMA.—Tumor giant cells appearing in reticulum cell sarcoma have been described by Oberling,<sup>28</sup> Warren and Picena,<sup>29</sup> Gall and Mallory,<sup>30</sup> Foot,<sup>31</sup> and many others. The cells are small for giant cells, usually measuring from 15 to 25 microns in diameter; their cytoplasm is customarily scant and faintly acidophilic in tissue sections, whereas the nucleus consists of a giant multilobulated structure occupying almost the entire cell body. The cells of Figs. 35 and 36 are of this type as seen in tissue sections. The nuclear membrane is distinct and the nuclear lobes are characteristically closely affixed to one another. The chromatin content is sparse, giving a pale vesicular appearance to the lobes, which makes even more prominent a large, round or irregular, basophilic nucleolus to be found usually one or two



# PLATE VII

- Fig. 35—Giant cell in section of lymph node in reticulum cell sarcoma. Hematoxylin and eosin stain, X2000 AIP Neg 97616
- Fig. 36—Giant cell in section of lymph node in reticulum cell sarcoma. Hematoxylin and eosin stain, X2000 AIP Neg 98016
- Fig. 37—Giant cell in imprint of lymph node in leukemic reticulo-endotheliosis. May-Grünwald-Giemsa stain, X2000 AIP Neg 97612
- Fig. 38—Giant cell in imprint of lymph node in leukemic reticulo-endotheliosis. May-Grünwald-Giemsa stain, X2000 AIP Neg 97611
- Fig. 39—Giant cell in imprint of lymph node in leukemic reticulo-endotheliosis. May-Grünwald-Giemsa stain, X2000 AIP Neg 97610
- Fig. 40—Giant cell in section of lymph node in leukemic reticulo-endotheliosis. Hematoxylin and eosin stain, X2000 AIP Neg. 97609
- Fig. 41—Atypical enlarged reticulum cell in leukemic reticulo-endotheliosis. Hematoxylin and eosin stain; X2000. AIP Neg. 97607

to a lobe. None of these forms contained the acidophilic nucleoli of the fully developed Reed-Sternberg cells as seen in tissue sections.

**D. GIANT CELLS IN LEUKEMIC RETICULO-ENDOTHELIOSIS.**—Cases of leukemia in which the leukemic process is centered primarily in the reticulo-endothelial cells as they exert their hematopoietic function, that is, cases characterized by leukemic reticulo-endothelial cells and their differentiation products, have been reported as such by Ewald<sup>32</sup> and Downey.<sup>33</sup> Since cases of this nature are usually included in the literature of the monocytic leukemias, the structure of the leukemic reticulum cells has received little attention from other writers. In several cases of leukemic reticulo-endotheliosis in the material available at the Institute, the leukemic reticulum cells or mesenchymal elements of the blood-forming organs were observed in the process of forming giant, bizarre, polymorphonuclear, or multinucleated reticulum cells similar to those found in reticulum cell sarcomas. In dry-fixed impressions of a lymph node in such a case, cells such as those of Figs. 37 to 39 are seen. The nuclei are round, oval or irregular in outline; the chromatin pattern consists of fine to moderate sized chromatin particles, irregularly arranged, but often sharply demarcated from the parachromatin. The nucleoli are basophilic and small but distinct. Three of the nuclei in Fig. 37 are within the main cytoplasmic mass, the other two apparently are within separate cytoplasmic divisions. The nuclear patterns are less leptochromatic than those of normal reticulum cells, and this may be explained in this case by the fact that differentiation was proceeding along lymphocytic and plasmacellular lines. The cytoplasm may be scant or abundant, and the basophilic spongioplasm is arranged in flakes against a colorless or yellowish hyaloplasm.

In tissue sections the nuclei of these reticular giant cells (Figs. 40 and 41) may possess pale or hyperchromatic patterns. The cell of Fig. 40 was the only one found which resembled the Reed-Sternberg cell; however, the basophilia of the large nucleolus in the one lobe, the hyperchromatism of the remaining lobes, and the small size of the nucleus are not characteristics of Reed-Sternberg cells. Compare these cells of leukemic reticulo-endotheliosis with the illustrations of Reed-Sternberg cells (Figs. 22 to 25) and those of the tumor giant cells of reticulum cell sarcoma (Figs. 35 and 36).

**E. GIANT CELLS IN METASTATIC TUMORS PRIMARY IN OTHER THAN HEMATOPOIETIC ORGANS.**—Metastatic undifferentiated carcinomas frequently simulate the appearance of primary affections of the blood-forming organs, particularly of the lymph nodes. The tumor giant cells of these metastases, then, must likewise

#### PLATE VIII

Fig. 42A.—Transitional cell carcinoma, metastatic in a section of lymph node. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97613.

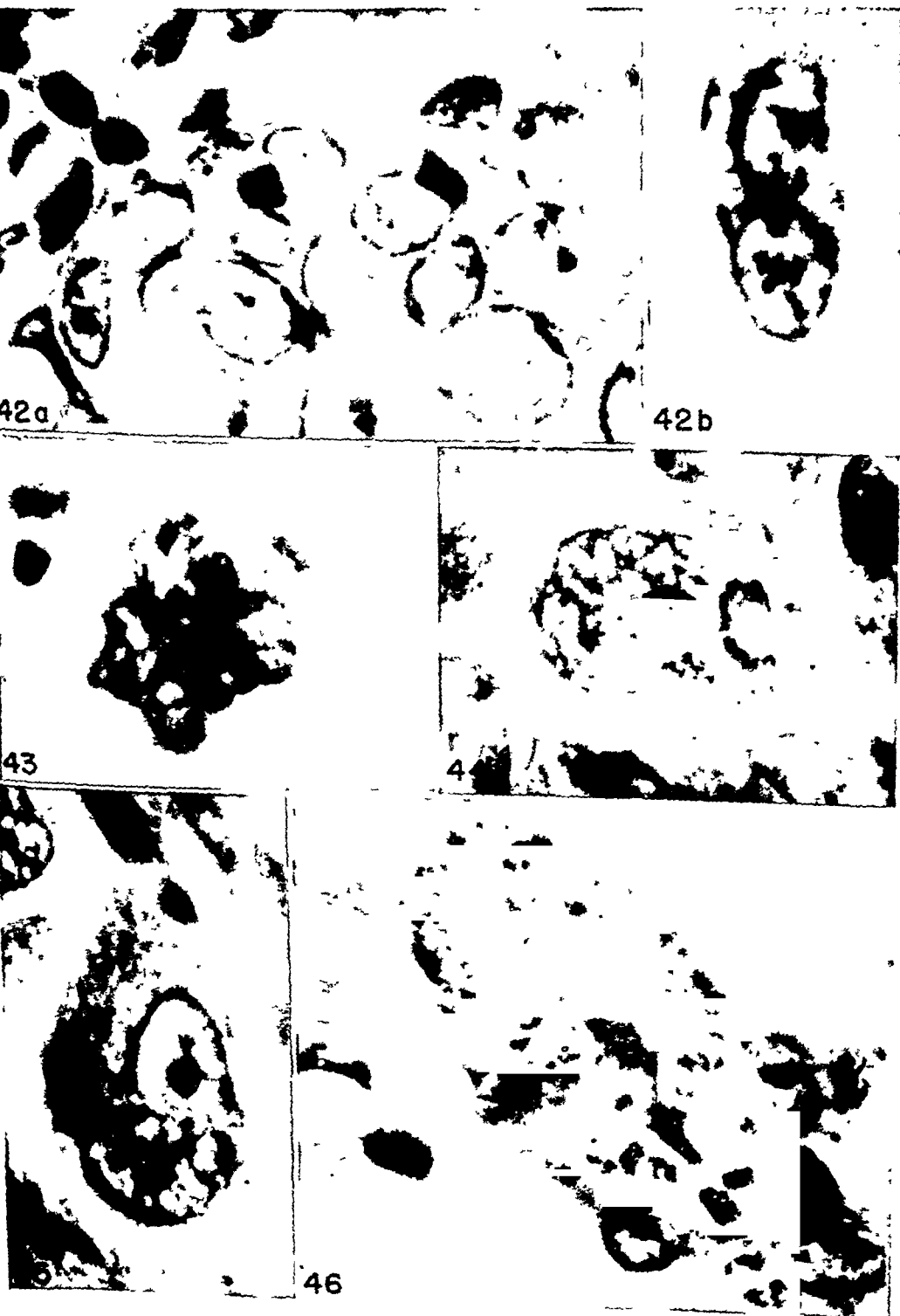
Fig. 42B.—Tumor giant cell, metastatic from breast in a section of lymph node. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97009.

Fig. 43.—Tumor giant cell, metastatic from breast in a section of lymph node. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97008.

Fig. 44.—Tumor cell in metastatic malignant melanoma in section of lymph node. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 98013.

Fig. 45.—Tumor giant cell in metastatic malignant melanoma in section of lymph node. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 98014.

Fig. 46.—Osteoclast in a section of tissue in osteitis fibrosa cystica with a parathyroid adenoma. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97906.





be differentiated from the giant cells in primary affections of the hematopoietic system. A discussion of three of the more common examples of these metastases follows.

1. *Transitional Cell Carcinoma*.—Transitional cell carcinoma (lympho-epithelioma) metastatic to lymph nodes often simulates reticulum cell sarcoma. In Fig. 42a is shown a cord of epithelial cells cut tangentially and apparently surrounded by lymphoid stroma in such a case. Although many of the epithelial characteristics of these cells have been lost, usually enough remain on which to base a definitive diagnosis. Although individual cellular outlines and intercellular bridges are missing, three distinguishing features are listed by Custer<sup>24</sup>: they are: overlapping of the nuclei, prominence and large size of many of the nucleoli, and the syncytial arrangement of the epithelial growth. The nuclei as seen in sections have a distinct nuclear membrane, scant, fine chromatin granules arranged in a delicate linen network, and a rather prominent, round or oval, acidophilic nucleolus. There is marked variation in individual nuclear size in any particular epithelial cord or nest. Ewing<sup>25</sup> points out the alveolar structure and lack of intercellular reticulum after silver staining.

2. *Anaplastic Carcinoma*.—Anaplastic carcinoma metastatic to lymph nodes may likewise possess morphologic features similar to reticulum cell sarcoma. Figs. 42b and 43 are illustrations of tumor giant cells of an anaplastic carcinoma of the breast metastatic in an axillary lymph node. Usually the functions and hence the special morphologic characteristics of the cytoplasm of the cells in the tissue of primary origin are lost and the cytoplasm is nonspecific, but occasionally special stains will bring out granules or secretory products helpful in the recognition of the true nature of these cells. Otherwise, the tumor giant cells cannot be characterized apart from the extreme diversity of types to be seen in a given lesion. Nuclear lobulations may be few or numerous, nuclear lobes small or large, nuclear outlines extremely bizarre, and nuclei pale and vesicular (Fig. 42b); the more prominent nucleoli are usually acidophilic (Fig. 43). Fortunately, the histopathologic pattern of the metastatic neoplasm, the sheets, cords, or nests of epithelial cells sequestered in the node, is in itself not difficult of recognition. The characteristics of such cells in dry-fixed preparations may be observed in Rohr's<sup>10</sup> monograph (his Figs. 120 and 121a) or as illustrated by Paseyro<sup>21</sup> (his Figs. 30, 33, and 99).

3. *Metastatic Malignant Melanoma*.—The tumor giant cells of the metastases in tumors of this type present the customary features of the tumor giant cells found in the general group of anaplastic malignancies. Bizarre nuclear outlines, scant or excessive chromatin content, absent or prominent basophilic nucleoli, and moderate or extreme lobulation of nuclei are to be found (our Figs. 44 and 45). Melanin granules may or may not be present within the alveolar type of acidophilic cytoplasm. The dopa reaction should be positive in these cell types. Dry-fixed preparations of similar cells are to be found in Paseyro's<sup>21</sup> monograph (his Figs. 31, 96, and 97). Strumia<sup>9</sup> depicts giant, phagocytic, hemohistioblastic cells laden with black pigment derived from broken down malignant melanoma cells (his Fig. 11 and Plate VIII).

F. GIANT CELLS RELATED TO THE SOFT TISSUES OF BONE.—The giant cells in conditions primarily affecting the soft tissues of bones are included in this discussion, not because of any desire to classify the inflammatory and neoplastic lesions of bones among the disorders of the blood-forming organs per se, but because recent studies of the giant cells in the inflammatory and neoplastic lesions of bones point increasingly to their origin from the same mesenchymal elements that are of intrinsic importance in the origin of primary hematopoietic disorders. It seems reasonable that the structure of such giant cells, falling as they do in a borderline zone between the fields of orthopedic pathology and hematopathology, should engage as much interest among hematopathologists as the reticulo-endothelial system does among orthopedic pathologists.

1. *Osteoclasts*.—These large, multinucleated cells make their appearance wherever bone is formed or undergoes a destructive process. They were first described by Kölliker<sup>5</sup> in 1872.

In tissue sections they are large multinucleated cells, measuring from 30 to 90 microns, with the longer diameter frequently parallel to a bone spicule but sometimes perpendicular to one. The nuclei are round or oval, are rather poor in chromatin, and possess a distinct nuclear membrane and one or two prominent, round, or rod-shaped (Fig. 46) nucleoli. The cytoplasm is abundant, deeply basophilic, granular or vacuolated, and provided with branching processes. Osteoclasts are usually found in Howship's lacunae, bays or grooves in dissolving bone (Fig. 46). Rustizky<sup>36</sup> said that they all contained calcium granules, but Maximow and Bloom<sup>18</sup> found neither bone salts nor cell remnants in their cytoplasm.

In dry-fixed smears or imprints the many round or oval nuclei of the osteoclast resemble the pattern of histiocytic or reticulum cell nuclei, although, again as in sections, one to three prominent, deeply basophilic nucleoli are observed (Fig. 47). The nuclear pattern is characterized by small angular distinct chromatin particles irregularly distributed throughout the nucleus. Here there is chromatin-parachromatin distinction and the nuclear membrane appears to be thinner than in section preparations. The cytoplasm immediately surrounding the nuclei is deeply basophilic and composed of a dense granular spongioplasm, whereas the more peripheral cytoplasm is composed of a background of slightly acidophilic hyaloplasm in which occasional flakes of basophilic spongioplasm appear. No azurophil granules are observed. Sabin and Miller<sup>37</sup> in their Fig. 5 show such an osteoclast in a fixed film of marrow, its cytoplasm filled with eosinophilic particles which stain like bone, and its nuclear configurations difficult to make out.

*Origin:* These cells arise from the mesenchymal elements of the bone marrow and periosteum, occasionally by fusion of osteoblasts.

*Function:* Most writers<sup>35, 37</sup> attribute a phagocytic function to the osteoclasts, placing them in the group of foreign body giant cells because of their constant relation to the resorption or dissolution of bone and their disappearance when the resorption of bone ceases in a particular site.

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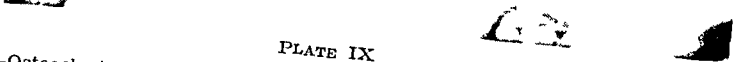


PLATE IX

Fig. 47.—Osteoclast in sternal bone marrow aspirate. May-Günwald-Giemsa stain,  $\times 2000$ . AIP Neg. 98023.

Fig. 48.—Multinucleated giant cell in a section of tissue in osteitis fibrosa cystica with a parathyroid adenoma. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97884.

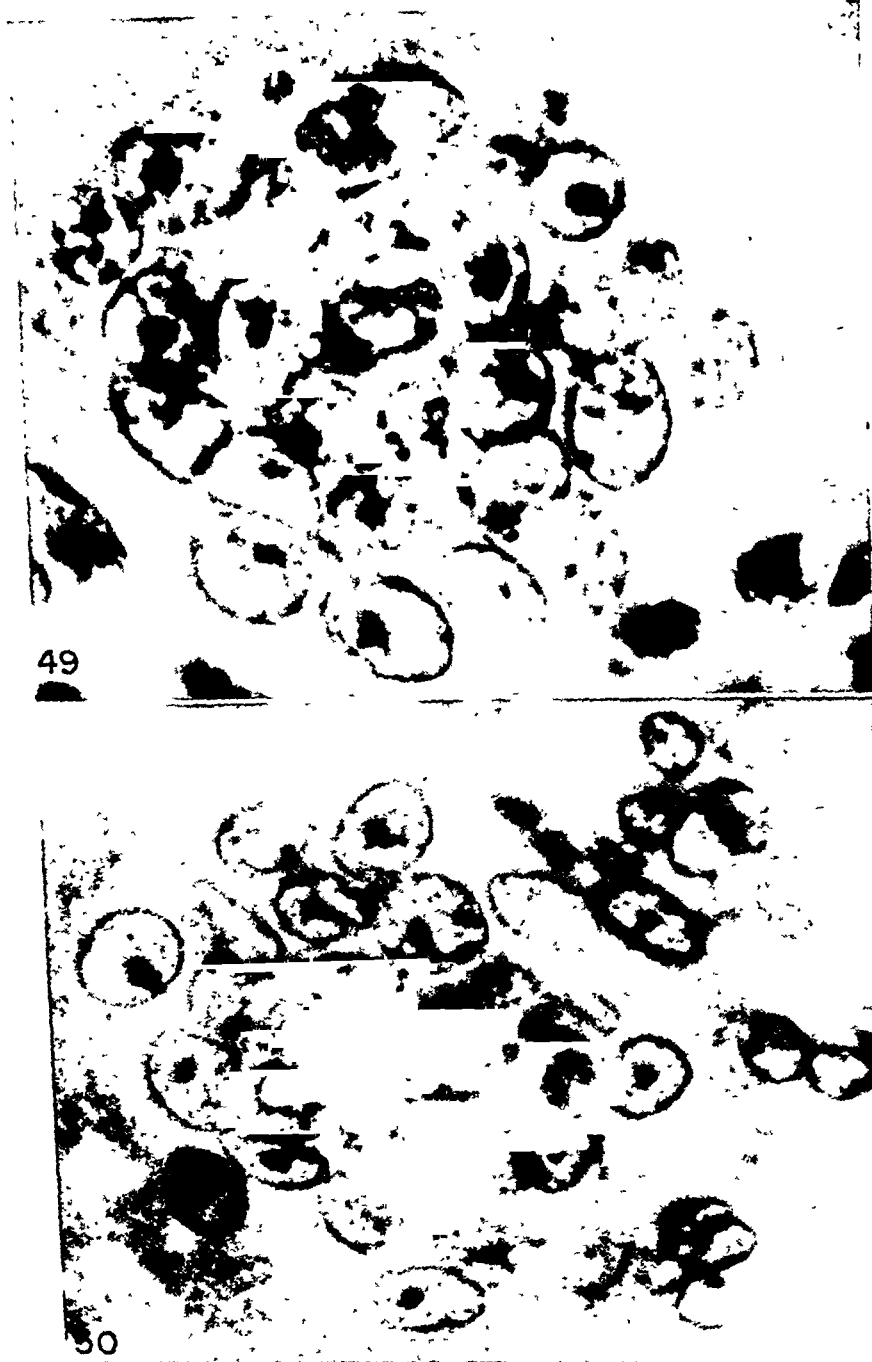


PLATE X

Fig. 49.—Multinucleated giant cell in a section of benign giant cell tumor of bone. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97894.

Fig. 50.—Multinucleated giant cell in a section of malignant giant cell tumor of bone. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97893.

Maximow and Bloom,<sup>18</sup> as mentioned before, finding no phagocytic inclusions or bone salts within osteoclasts, hold that there is slight possibility of such cells possessing a phagocytic function.

Dameshek and Miller<sup>7</sup> state that the polykaryocyte, precursor in a relatively uncommon type of megakaryocytic formation, is probably identical with osteoclast and forms the adult megakaryocyte by nuclear fusion. (Comparison should be made of their Fig. 5, our Figs. 10 and 47, and Rosenthal's<sup>16</sup> Figs. 11 and 13.)

2. *Giant Cells of Bone in Osteitis Fibrosa Cystica Due to Adenoma of the Parathyroid Glands.*—Osteoclasts are numerous about the bony spicules in the characteristic lesions of osteitis fibrosa cystica (our Fig. 46). Giant cells, indistinguishable from osteoclasts, are likewise numerous in the fibrous connective tissue apart from the bony spicules or in and about foci of hemorrhage (Fig. 48). However, ingested red corpuscles are not observed within the cell bodies.

3. *The Giant Cells of Benign Giant Cell Tumor of Bone.*—Numerous giant cells are irregularly distributed through a benign stromal background of connective tissue in this condition. They have to all appearances the same morphologic characteristics as osteoclasts in tissue sections (Fig. 49), but they lack the relationship to Howship's lacunae. Their appearance in imprint preparations has not been described. Many modern writers consider these giant cells as osteoclasts or foreign body giant cells, not as tumor cells. Ewing,<sup>35</sup> however, emphasized giant cell proliferation as an essential feature of the lesion to which he referred as "osteoclastoma." Geschickter and Copeland<sup>38, 39</sup> ascribe these giant cells to abnormal hyperplasia of osteoclasts, whereas Jaffe and associates<sup>40</sup> state that these giant cells are not osteoclasts and that they possess definite differences from osteoclasts.

*Origin:* These cells probably arise from mesenchymal elements<sup>40</sup> (histiocytes, reticulum cells); however, because these cells at times apparently form a portion of the lining of the blood vessels, an endothelial origin has been claimed, although it should be recalled that many of the simple vascular channels within bone are lined by littoral reticulum cells and not by simple endothelium. Foot<sup>31</sup> tends to favor the idea of a reticulo-endothelial origin of these cells and notes that although the giant cells usually lie in clear spaces amid the reticular network, remnants of argyrophil substance may be found within their cytoplasm.

4. *The Giant Cells in Malignant Giant Cell Tumor of Bone.*—The stromal background of connective tissue is neoplastic both in structure and functions. The multinucleated giant cells irregularly distributed throughout these lesions (Fig. 50) present few or no characteristic features which distinguish them from osteoclasts. Their appearance in dry-fixed impressions has not been described. However, Jaffe and associates<sup>40</sup> observed that the nuclei of these giant cells actually may share in the atypism of the stromal cells (their Fig. 4) and become swollen and large, out of proportion to cell size. It is important to note that these cells are almost always absent in the metastatic lesions of this tumor. There is but one case in the files of the Institute of Pathology in which the same "benign" appearing, multinucleated giant cells were found in an extension or

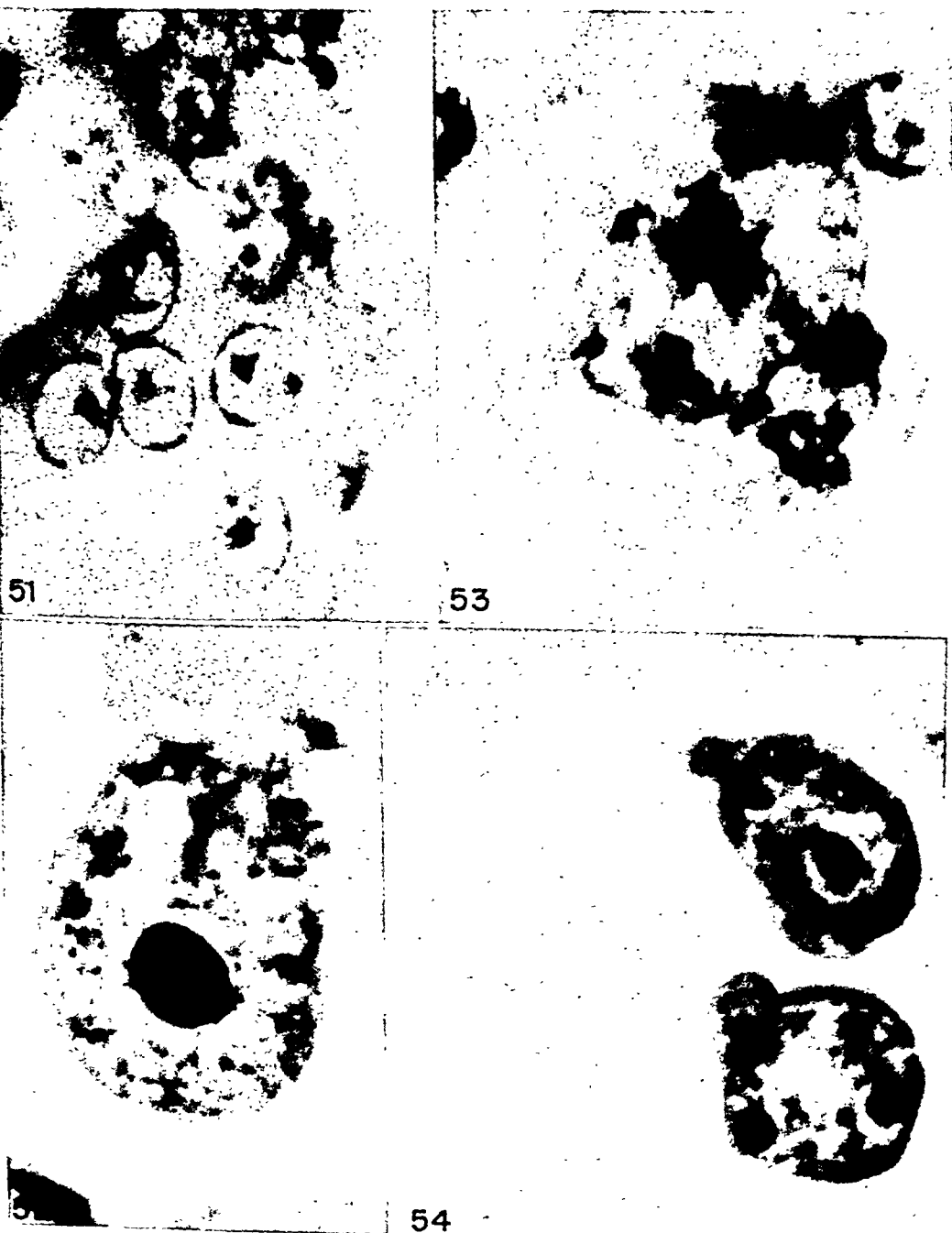


PLATE XI

Fig. 51.—A portion of a multinucleated giant cell in a section of osteogenic sarcoma. Hematoxylin and eosin stain; X2000. AIP Neg. 97890.

Fig. 52.—Mononuclear tumor giant cell in a section of osteogenic sarcoma. Hematoxylin and eosin stain; X2000. AIP Neg. 97889.

Fig. 53.—Multilobulated tumor giant cell in a section of osteogenic sarcoma. Hematoxylin and eosin stain; X2000. AIP Neg. 97892.

Fig. 54.—Binucleated tumor giant cell in a section of osteogenic sarcoma. Hematoxylin and eosin stain; X2000. AIP Neg. 97891.

spread from the tumor in the ulna to an epitrochlear lymph node. Our Fig. 50 is from the ulnar lesion in this case.

5. *Giant Cells in Osteogenic Sarcoma.*—In many instances tumor giant cells of all sizes and shapes are abundant in this lesion. These tumor giant cells are to be distinguished both from the foreign body giant cells which may also be present, particularly about areas of hemorrhage, and from the osteoclasts in focal areas of bone resorption. Depicted in Fig. 51 is a giant cell of this reactive type from an osteogenic sarcoma. The tumor giant cells of osteogenic sarcoma may be giant mononuclear forms (Fig. 52), polymorphonuclear forms (Fig. 53), or multinucleated forms (Fig. 54). The extremely large size of the nuclei may be appreciated at once by comparison with those of any of the osteoclasts previously described (Figs. 47 and 48). The nuclei are bizarre in shape, frequently hyperchromatic with coarse chromatin clumps in evidence (Figs. 52 and 54). One or more giant, round or irregular, acidophilic nucleoli are present. The cytoplasm may be scant or abundant and shares in the cytoplasmic type of the more numerous, surrounding, smaller neoplastic cells as to intrinsic structure and nature of intercellular substance. Bizarre, atypical, occasionally multipolar mitoses are to be found in these cases. The morphologic characteristics of the cell in dry-fixed preparations have not been described.

*Origin:* Tumor giant cells arise by abnormal mitoses in or fusion of tumor cells. Ultimately they arise from the embryonic or mesenchymal elements capable of bone formation.

6. *Giant Cells in Ewing's Tumor of Bone.*—Ewing<sup>35</sup> mentioned an occasional report of the presence of giant cells in the tumor known by his name. This observation is of importance to the hematologist in view of Piney and Hamilton-Paterson's<sup>41</sup> description of giant, histiocyte-like elements, which they call "dysmorphokaryocytes." These cells, which possess indented nuclei and irregular hyaline cytoplasm, may be found scattered among the cells of the sternal marrow prior to any other indication of the spread of the tumor from its primary state. No example of this cell type was to be found in the material which we have studied. Rohr<sup>10</sup> states that in his hands sternal punctures have revealed no sure signs of malignant changes in cases of this disease.

*Origin:* Ewing<sup>35</sup>, p. 368 suggested as the cellular origin of his tumor "the perivascular endothelium," which to him represented a perivascular cell with mere endothelial potentialities. Oberling and Raileanu<sup>42</sup> and more recently Stout<sup>43</sup> would prefer to designate this tumor as a variant of reticulum cell sarcoma on the basis of the demonstration of reticulum cells as integral parts of some of them. Stout does not mention the presence of giant cells in this tumor.

7. *Giant Cells in Other Conditions.*—Giant cells identical with or similar to the osteoclast or the foreign body type may also be found in healing fractures, exuberant callus formation, acute and chronic osteomyelitis, rickets, osteomalacia, osteogenesis imperfecta, osteochondritis desiccans, Paget's disease, angioma, fibrous dysplasia of bone, medullary fibroma, xanthofibroma, and medullary chondroma, to mention but a few conditions affecting the soft tissue of the bone and not ordinarily the province of the hematopathologist.

## G. PLASMACELLULAR GIANT CELLS.—

1. *Multinucleated Plasma Cells*.—Multinucleated plasma cells usually are present wherever the ordinary mononuclear plasma cells are to be found in the blood-forming organs. They were described first by Cajal (Michels<sup>44</sup>). They are not uncommon in practically any of the subacute and chronic inflammatory reactions as well as in agranulocytosis and aplastic anemia (Rohr's<sup>10</sup> Figs. 22 and 71).

In stained tissue sections they are smaller than most giant cells, being about 20 microns in diameter, round, oval or elongated, and possessing from 2 to 4 discrete nuclei usually placed near the periphery of the cell. At times the nuclear pattern may be rather characteristic, consisting of coarse particulate chromatin arranged about the lining of the nuclear membrane in a spokelike fashion so as to present Pappenheim's "radkern" effect; in numerous instances the pattern may merely resemble the lymphocytic nuclear pattern with somewhat coarser features. A nucleolus usually is present. The cytoplasm is occasionally vacuolated and is deeply basophilic, save for a central pale area near the nuclei which is a rather specific characteristic. When stained with Pappenheim's methyl green-pyronin the cytoplasm is a brilliant red whereas the chromatin is a greenish purple (Slider and Downey<sup>2</sup>).

In dry-fixed impressions the nuclei are round or oval and possess a chromatin pattern midway between those of the reticulum cell and the lymphocyte, in that the chromatin granules are arranged in coarse clumps but present chromatin-parachromatin distinction (our Fig. 55). Characteristically in these preparations the "hof" or central light area has disappointingly vague borders and the cytoplasm is found to consist of closely packed, deeply basophilic granules or flakes at the periphery; these become increasingly scant as the center of the cell is approached, allowing more and more of the pale pink hyaloplasm to show through in the central cytoplasmic area. These cells are devoid of azurophil granules.

*Origin*: These cells arise from pre-existing plasma cells by amitotic nuclear division without cytoplasmic division (Michels<sup>44</sup>) ultimately from reticulum cells or lymphocytes.

2. *Multinucleated Myeloma Cells*.—Because of our lack of knowledge as to the function of the plasma cell, it is difficult to affirm or deny Rohr's<sup>10</sup> contention that multiple myeloma represents a malignant tumor of the reticulum. However, certain differences in structure between the cells of this and the preceding group can be noted at times.

These cells as seen in tissue sections are usually merely binucleate, but 3 to 8 or even more discrete nuclei have been observed. They may resemble plasma cells with their round, lymphocyte-like, peripheral nuclei and abundant basophilic cytoplasm with a central light area (Fig. 56), or they may present all transitions to larger cells with more abundant colorless or slightly acidophilic cytoplasm, the pale perinuclear area thus being lost. Ewing<sup>25</sup> observed mitoses in these cells but less frequently as they approached the plasma cell type. According to Jones,<sup>22</sup> myeloma cells do not take the specific methyl green-pyronin stain described for plasma cells.



In dry-fixed preparations the discrete nuclei may vary in size within a single cell and in different cases may show nuclear patterns of the reticulum cell, the plasma cell, or the lymphocyte. These cells may possess as many as from 5 to 8 nuclei which may be plasmacellular in type, but each nucleus contains a moderate-sized, dark blue nucleolus. The cytoplasm may contain less basophilic spongioplasm than the cell of Fig. 55 and a few scattered azurophil granules may be found within the central cytoplasmic area. In Fig. 57 the pattern of the two nuclei resembles that of a reticulum cell with prominent basophilic nucleoli, and the cytoplasm is composed of diffusely granular basophilic spongioplasm. Further illustrations of giant cells of this group are to be found in Rohr's<sup>10</sup> monograph (his Fig. 116) and in a recent work by Erf and Herbut,<sup>45</sup> who further attest to the value of the dry-fixed preparations as an adjunct to tissue sections in cytologic diagnosis.

*Origin:* Multinucleated myeloma cells may arise from the myeloblast (Fleischhaecker and Klima<sup>46</sup>), and from the lymphocyte (Ewing<sup>35</sup>) or from the reticulum cell (Rohr<sup>10</sup>). Recently, Herbut and Erf<sup>47</sup> have described megakaryocytoid and lipoblastic giant myeloma cells in cases of multiple myeloma.

*3. Giant Cells of Plasma Cell Leukemia.*—Plasma cell leukemia is generally regarded as closely related to multiple myeloma (Lubarsch,<sup>48</sup> Moss and Ackerman,<sup>49</sup> Patek and Castle,<sup>50</sup> Jackson and associates<sup>51</sup>). Giant cells are less frequent than in multiple myeloma and, when they occur, rarely possess more than two nuclei (our Fig. 58). Such a cell from a dry-fixed bone marrow aspirate is depicted in Fig. 58. In structure it tends to resemble ordinary reactive multinucleated plasma cells more closely than the comparable myeloma cell. Patek and Castle<sup>50</sup> using the same technique depict a similar cell (their Fig. 2) and Osgood and Hunter<sup>52</sup> portray in colors (their Fig. 8) just such a cell with one of the nuclei very large and irregular and the cytoplasm containing azurophil granules.

## H. GIANT CELLS IN SYSTEMIC DISTURBANCES INVOLVING LIPIDS.—

*1. Giant Cells in Niemann-Pick's Disease.*—The giant cells in Niemann-Pick's disease are relatively rare, the mononuclear type predominating. According to Pick<sup>53</sup> these cells "include one or two small nuclei, occasionally many." The central cell of our Fig. 59, as seen in tissue section, contains three such nuclei. The nuclei are round or oval, peripheral and discrete, and the chromatin particles are small but numerous, so that the pattern is similar to that of the nucleus of a reticulum cell or a histiocyte. The abundant cytoplasm con-

### PLATE XII

Fig. 55.—Multinucleated plasma cell in sternal bone marrow aspirate. May-Grünwald-Giemsa stain; X2000. AIP Neg. 97882.

Fig. 56.—Binucleated myeloma cell in a section of tumor in multiple myeloma. Hematoxylin and eosin stain; X2000. AIP Neg. 98131.

Fig. 57.—Binucleated myeloma cell in a bone marrow aspirate in multiple myeloma. May-Grünwald-Giemsa stain; X2000. AIP Neg. 98132.

Fig. 58.—Binucleated plasma cell in sternal bone marrow smear in plasma cell leukemia. Dr. R. P. Custer's case. May-Grünwald-Giemsa stain; X2000. AIP Neg. 98129.

Fig. 59.—Multinucleated Niemann-Pick cells in a section of spleen. Hematoxylin and eosin stain; X2000. AIP Neg. 97888.

Fig. 60.—Multinucleated Gaucher's cell in sternal bone marrow aspirate. Dr. R. P. Custer's case. May-Grünwald-Giemsa stain; X2000. AIP Neg. 97881.

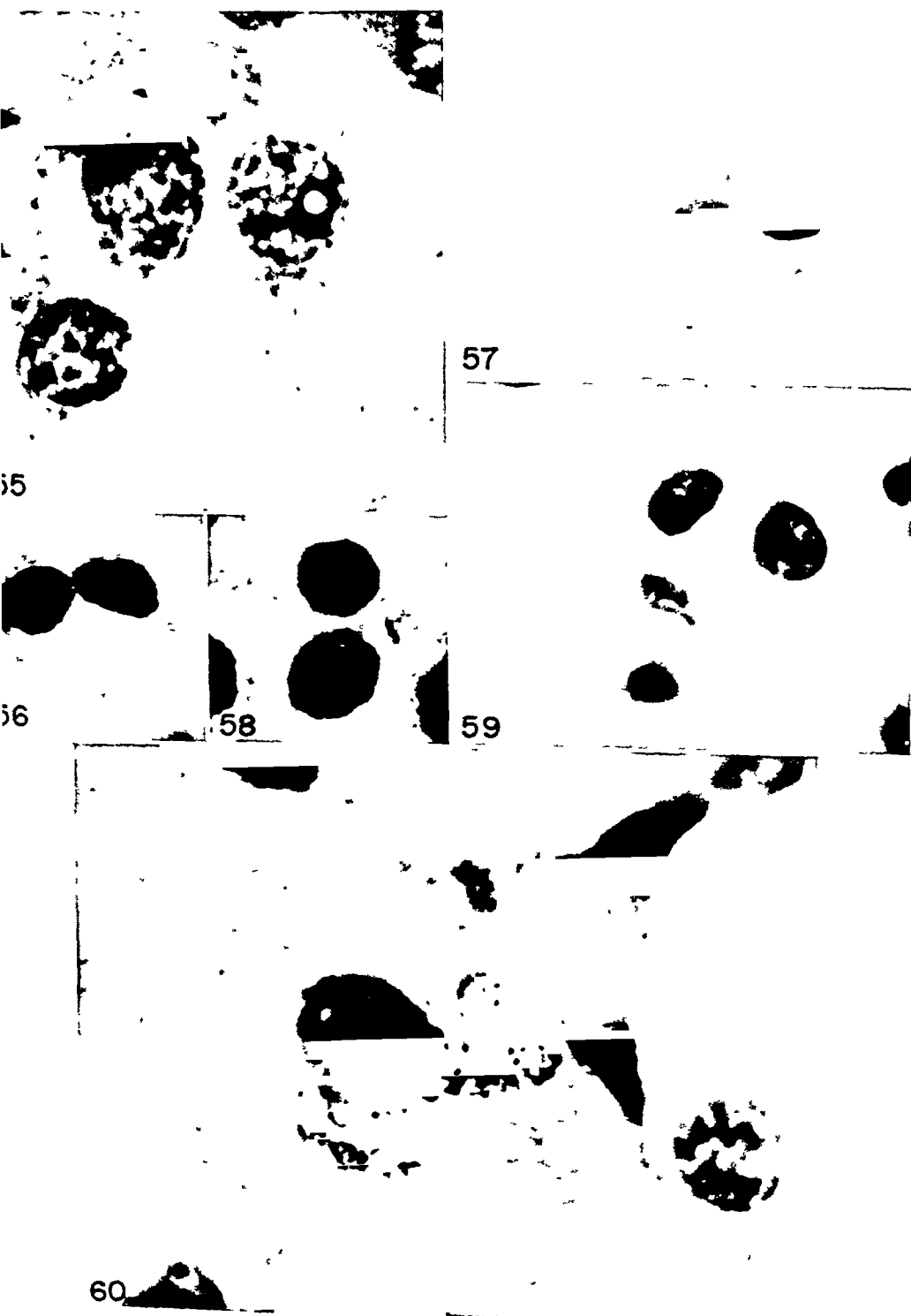


Plate XII

(For legend, see opposite page.)

sists merely of small and large, round vacuoles with thin intervening septa. The vacuoles are clear with ordinary staining methods, stain poorly (depending on mordant) with Nile blue and sudan III, but stain with the Smith-Dietrich method. The cells contain a phosphatide, sphingomyelin (Klenk<sup>54</sup>). For further cytologic detail and the histogenesis of this cell type the reader is referred to Bloom's<sup>55</sup> detailed monograph. The characteristics of these cells in dry-fixed preparations are well illustrated in photomicrographs of Pick<sup>53</sup> (his Fig. 1), in which the cells from the spleen contain a dense homogeneous central cytoplasm with the typical vacuoles peripherally situated, and of Rowland<sup>56</sup> (his Figs. 47 and 48), in which cells from the spleen and bone marrow show a more diffuse distribution of the vacuoles.

2. *Giant Cells in Gaucher's Disease.*—Giant cells are rather frequently found in Gaucher's disease although the mononuclear type still predominates. Such Gaucher cells are multinucleated. As seen in tissue sections, the discrete nuclei have reticulum cell or histiocytic characteristics although the chromatin content is more conspicuous. The cytoplasm stains poorly with sudan III, Smith-Dietrich, or Nile blue; however, Mallory's aniline blue, connective tissue stain reveals a cytoplasm filled with a dark blue fibrillar substance, weblike in arrangement, in contrast to the honeycombed or foamlike appearance of other lipid-containing cells. In fact, the cytoplasm of Gaucher cells is frequently spoken of as being wrinkled. Piney and Hamilton-Paterson<sup>41</sup> state that the fibrils of these cells may be brought out by silver impregnation. The appearance of these cells in tissue sections is well portrayed by Mandelbaum and Downey.<sup>57</sup> Most authors state that the cells contain the cerebroside, cerasin, originally described by Lieb.<sup>58</sup>

The appearance of multinucleated Gaucher cells in dry-fixed preparations is similarly characteristic. The cells measure more than 50 microns in diameter. The oval or round discrete nuclei are relatively small and resemble reticulum cell nuclei, save that the irregular angular pieces of chromatin are coarser, although the chromatin-parachromatin distinction is preserved. A single, small, round basophilic nucleolus is present in each nucleus. Basophilic fibrils are to be found embedded in the colorless or faintly basophilic cytoplasm (our Fig. 60). As degeneration sets in, however, vacuoles may also appear in the cytoplasm of these cells.

3. *Giant Cells in Hand-Schüller-Christian Disease.*—The majority of the giant cells seen in the xanthomatous lesions of this disease are lipid-containing but otherwise ordinary foreign body giant cells. The cytoplasm stains well with sudan III. The cells contain cholesterol and cholesterol esters as described originally by Pinkus and Pick<sup>59</sup> in symptomatic xanthomatoses. A more spectacular type of giant cell called the Touton giant cell<sup>60</sup> (Fig. 61) is occasionally found. This cell presents a rim of closely spaced discrete nuclei of the histiocytic type about an area of dense, homogenous, acidophilic cytoplasm. The ring of nuclei in turn is surrounded by an abundant, peripheral, foamy cytoplasm. It must be emphasized that cells of this type are not specific for Hand-Schüller-Christian disease but may be found in the so-called secondary xanthomatoses as well; in fact, the cell depicted in Fig. 61 was found in an area of fatty degeneration within a kidney, the seat of chronic pyelonephritis. The structure

of the so-called nonspecific lipoid histiocytes is apparently not well known; Paseyro<sup>21</sup> (his Fig. 113) depicts such a large vacuolated cell from the bone marrow in a case of diabetic xanthomatosis. For further interesting details the reader is referred to the monograph of Thannhauser and Magendantz<sup>21</sup> upon this subject.

*4. Normal Fat Cells.*—Normal fat cells within the blood-forming organs present few structural features that are of differential significance as seen in tissue sections. However, in dry-fixed preparations these large cells become flattened and distorted; they measure more than 60 microns in diameter and are usually round with an eccentric nucleus of the histiocytic type and an abundant colorless cytoplasm. As the cell body is broken up the cytoplasm takes on a wrinkled appearance and a faint basophilic tinge; at the same time numerous small vacuoles appear, particularly about the nucleus (our Fig. 62).

*5. Nonspecific, Fat-Containing Foreign Body Types of Giant Cells.*—These cells are not uncommonly found in the blood-forming organs, particularly the lymph nodes, under many and diversified conditions (Fig. 63). They are frequently seen in or about reactive or degenerative processes.

**I. GIANT CELLS IN MEASLES (Warthin-Finkeldey Giant Cells).**—These giant cells are variously found in the germinal centers and surrounding lymphoid tissue of the abdominal lymph nodes, spleen, tonsils, appendix, or lymphoid tissues of the respiratory and gastrointestinal tracts, usually in the prodromal period of measles. Giant cells were first observed in measles by Alagna<sup>62</sup> who believed they somewhat resembled megakaryocytes. Warthin<sup>63</sup> and Finkeldey<sup>64, 65</sup> gave the first accurate descriptions of this specific giant cell type. They are large syncytial multinucleated giant cells containing from 5 to 50 or more nuclei. The overlapping nuclei are generally arranged in a grape-like or mulberry-shaped cluster bordered by a scant rim of eosinophilic cytoplasm. In the germinal and reaction centers the nuclei tend to be of the pale, vesicular, reticulum cell type (Fig. 64), with several small- or moderate-sized nucleoli. In the surrounding lymphoid tissue are numerous similar cells (Fig. 65) with nuclei containing coarser chromatin particles resembling plasma cell nuclei. The nuclei in this latter cell type are somewhat more diffusely arranged, but the cytoplasm remains as a scant acidophilic border. Warthin<sup>63</sup> found no intracellular microorganisms or inclusions within the cytoplasm of these cells, but Hathaway<sup>66</sup> described them as being phagocytic.

*Origin:* Warthin<sup>63</sup> regarded these giant cells as derived from cells of the lymphoblast type. Mulligan<sup>67</sup> concluded that they arose by a "polynuclear abnormal development of the stem cell." The consensus is that they arise by amitotic division of reticulum cells, mesenchymal cells, plasma cells, or lymphocytes or by fusion of lymphocytes (Corbett<sup>68</sup>). In Fig. 66 is depicted the origin of such a cell in a distorted, reactive, multinucleated reticulum cell.

**J. FOREIGN BODY TYPES OF GIANT CELLS (LANGHANS' GIANT CELL).**—In 1868 Langhans<sup>69</sup> described the cell in tuberculosis which now bears his name. It is a multinucleated giant cell having from 2 to more than 1,000 round or oval nuclei. In tissue sections the nuclei contain fine chromatin particles and a small



PLATE XIII

Fig 61—Touton giant cell in section of kidney with fatty degeneration in chronic pyelonephritis Hematoxylin and eosin stain, X2000 AIP Neg 98138

Fig 62—Distorted fat cells in sternal bone marrow aspirate May-Grünwald-Giemsa stain, X2000 AIP Neg 97883

Fig 63—Fat-containing foreign body giant cell in section of reactive omental lymph node Hematoxylin and eosin stain X2000 AIP Neg 97001



PLATE XIV

Fig. 64.—Warthin-Finkeldey giant cell in section of lymphoid tissue of appendix in prodromal stage of measles. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 96999.

Fig. 65.—Warthin-Finkeldey giant cell in section of lymphoid tissue of appendix in prodromal stage of measles. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 96994.

Fig. 66.—Atypical enlarged reticulum cell in section of lymphoid tissue of appendix in prodromal stage of measles. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 96997.

Fig. 67.—Foreign body giant cell containing masses of *mycobacterium leprae* in section of lymph node in leprosy. Kinyoun's stain;  $\times 2000$ . AIP Neg. 97252.

Fig. 68.—Foreign body giant cell containing asteroid in section of lymph node in leprosy. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97250.

nucleolus or two; they resemble the nuclei of histiocytes or reticulum cells. By usage "Langhans' type giant cell" has come to designate the type of cell with its nuclei arranged in a ring near the periphery of the cytoplasm. The cell outline may be round, oval, irregular, or stellate. The cytoplasm itself is slightly acidophilic and may be finely granular or homogeneous. At times ingested inclusions may be present, that is *Mycobacterium tuberculosis* may be demonstrated by acid-fast methods. Actually, however, Langhans not only described the cells with the peripheral ring of nuclei but also those with nuclei at one or both poles of the cell body, scattered diffusely throughout its substance or placed centrally. This group of cells without the ring of nuclei is referred to as the foreign body type of giant cells by modern writers. Actually both groups are nonspecific. In infectious granulomas the so-called Langhans' type may be more frequently observed, whereas the foreign body type of nuclear arrangement is more likely to be found about inert foreign substances, although this is by no means invariable.

Foreign body giant cells arise by fusion of individual macrophages (histiocytes) or by amitotic division of their nuclei without constriction of the cell bodies. The macrophages in turn arise from lymphocytes and monocytes which have migrated from the blood or from cells of the fixed connective tissues possessing phagocytic abilities. The blood-forming organs, abounding as they do in fixed connective tissue cells with the ability of becoming phagocytic, usually draw upon this source for production of their phagocytic giant cells. It is beyond the scope of this review to include all of the affections of the blood-forming organs in which the presence of the various foreign body giant cells may be observed; however, some of them are herein listed:

1. Tuberculosis (Langhans<sup>69</sup>).
2. Syphilis, especially in the secondary stage (Michelson<sup>70</sup>), but not confined to that stage.

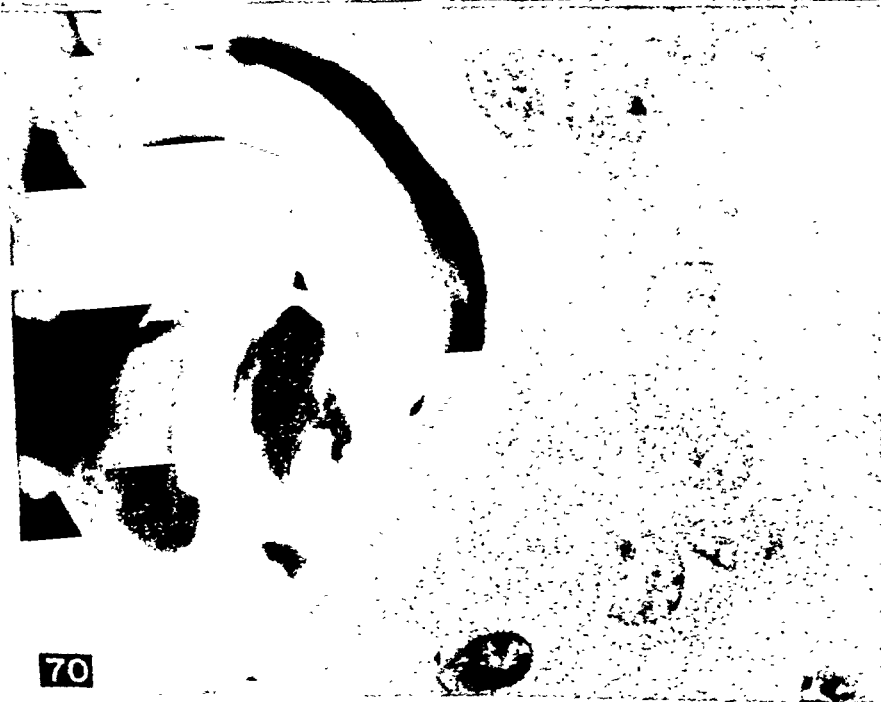
3. Actinomycosis (Forbus<sup>71</sup> in his Fig. 349 depicts a rod-shaped sulfur granule of *Actinomyces bovis* being ingested by a foreign body giant cell).

4. Leprosy. Langhans' and foreign body giant cells may be present in affected lymph nodes (our Figs. 67 and 68). With acid-fast techniques some of these cells are found to contain large numbers of *Mycobacterium leprae* arranged as packets and globi (Fig. 67). An additional finding, although not specific for leprosy, is the peculiar structures occasionally observed within the cytoplasm of such cells, called "asteroids" (Fig. 68). These were first described by Wolbach.<sup>72</sup> They are embedded in a round, pale staining area of the cytoplasm and consist of a central dense zone from which radiate from 10 to 15 or more sharp or blunt spindles, the entire structure being more or less star shaped. Asteroids are usually acidophilic and have been observed in the giant cells of tuberculosis, Boeck's sarcoid, and histoplasmosis as well as of leprosy, to mention but a few.

5. Lymphogranuloma venereum (lymphopathia venereum). In Fig. 69 is depicted a giant cell of the Langhans' type in the zone of palisaded histiocytes about a typical stellate abscess in an inguinal lymph node in this disease (Ash and Spitz<sup>73</sup>).



69



70

## PLATE XV

Fig. 69.—Langhans' type giant cell in section of inguinal lymph node in lymphogranuloma venereum (lymphopathia venereum). Hematoxylin and eosin stain; X2000. AIP Neg. 97620.

Fig. 70.—A portion of a large foreign body giant cell containing a Schaumann body in section of lymph node in Boeck's sarcoid. Hematoxylin and eosin stain; X2000. AIP Neg. 97619.



6. Visceral leishmaniasis, Kala-azar (Meleney<sup>71</sup>).

7. Boeck's sarcoid (Schaumann<sup>72</sup>). Foreign body giant cells are frequently observed in the granulomatous lesions of this disease. Rare cells may contain a large, laminated, deeply basophilic, roughly spherical structure called the "Schaumann body" (Fig. 70). These bodies are rather specific for Boeck's sarcoid although similar structures are occasionally to be found in other conditions.

8. Coccidioidomycosis. Shown in Fig. 71 is a giant cell which contains the causative organism, *Coccidioides immitis*, a nonbudding cell form undergoing endosporulation. Note the thick, double-contoured capsule.

9. Paracoccidioidal granuloma, South American blastomycosis (Moore<sup>76</sup>).

10. Histoplasmosis. In Fig. 72 is depicted an irregular type of foreign body giant cell in which numerous organisms are to be found. *Histoplasma capsulatum* appears as a very small yeastlike cell with a prominent nucleus and a thick refractile capsule. The small portion of free cytoplasm immediately above the giant cell in Fig. 72 likewise contains several such organisms.

As mentioned in the section on the giant cells in Hodgkin's disease, it should be kept in mind that nonspecific and merely reactive foreign body giant cells may occur in lymphomas and related afflictions of the reticulo-endothelial system.

For further details of the morphology and occurrence of foreign body giant cells in specific diseases the reader is referred to the excellent monographs of Haythorn<sup>1</sup> and Ash and Spitz.<sup>73</sup>

#### K. MISCELLANEOUS GIANT CELLS AND STRUCTURES RESEMBLING GIANT CELLS.—

1. *Nonspecific Reactive Giant Cells*.—Frequently in the nonspecific subacute and chronic inflammations affecting particularly the lymph nodes and spleen, a few reactive forms of giant cells are found of which little previous mention has been made in the literature. They consist of small foreign body giant cells with a few irregularly situated histiocytic nuclei, or they are polylobular mononuclear forms (Fig. 73), probably representing a reactive multilobulation of the reticulum cell nucleus. Their nuclei have small basophilic nucleoli and varying amounts of granular chromatin. The cell outline is usually irregular and the faintly acidophilic cytoplasm is not remarkable. Occasionally many fat-con-

#### PLATE XVI

Fig. 71.—A portion of a large foreign body giant cell containing *Coccidioides immitis* in section of lymph node in coccidioidomycosis. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97248.

Fig. 72.—Foreign body giant cell and detached cytoplasm containing *Histoplasma capsulatum* in section of lymph node in histoplasmosis. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97886.

Fig. 73.—Atypical enlarged reticulum cell in section of reactive lymph node. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97007.

Fig. 74.—Giant cell in section of lymph node in brucellosis. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 98135.

Fig. 75.—Giant cell in section of lymph node in disseminated lupus erythematosus. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 98130.

Fig. 76.—Phagocytic histiocyte in section of spleen in idiopathic pulmonary fibrosis. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97011.

Fig. 77.—Modified capillary in section of lymph node. Hematoxylin and eosin stain;  $\times 2000$ . AIP Neg. 97617.

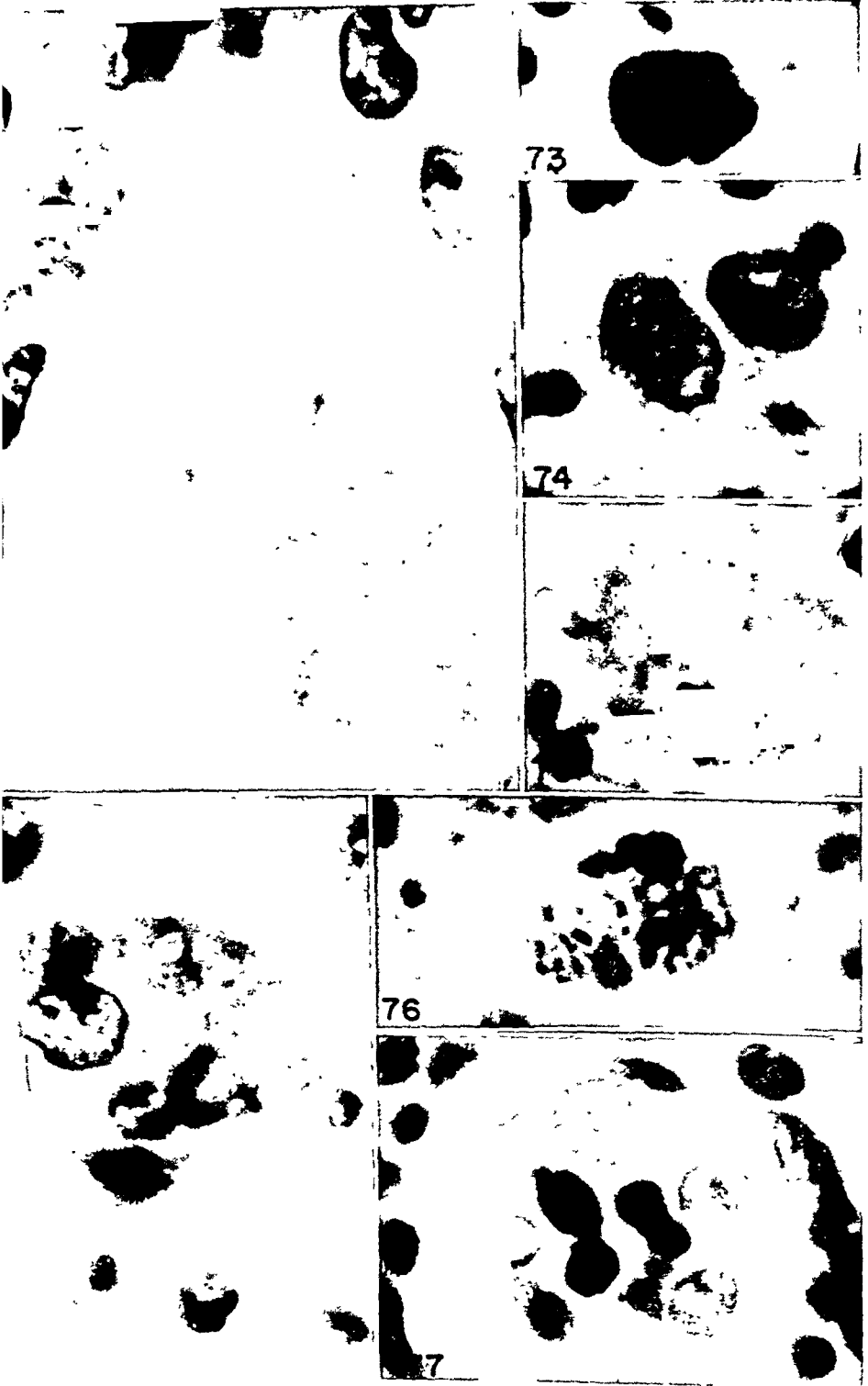


Plate XVI  
(For legend, see opposite page.)

taining vacuoles are to be found within the cell body (Fig. 63). These types are of importance because they have at times been confused with megakaryocytes or with the giant cells of Hodgkin's disease and reticulum cell sarcoma.

2. *The Giant Cells in Brucellosis.*—In certain relatively rare reactions of the blood-forming organs to brucella, particularly *Brucella melitensis*, the histiocytic and reticulum cell reaction may present nonspecific pleomorphism similar to that described in the preceding paragraph. Represented in Fig. 74 is just such a type of giant cell in the lymph node from a case of brucellosis proved by both blood cultures and recovery of the organism after animal inoculation. These cells have been spoken of as simulating the cells of Hodgkin's disease. However it is apparent that the nuclei of the giant cells of brucellosis are like those of nonspecific histiocytes or reticulum cells, with small although circumscribed basophilic nucleoli.

3. *The Giant Cells in the Lymph Nodes of Disseminated Lupus Erythematosus.*—Recently Fox and Rosahn<sup>17</sup> have described a type of giant cell frequently found in the lymph nodes in cases of disseminated lupus erythematosus. It is superficially somewhat like a megakaryocyte, possessing one or two large multilobular nuclei with a greater chromatin content than the customary reticulum cell nucleus (Fig. 75). Unlike a megakaryocyte, however, it lacks evidence of pseudopodial platelet formation, its cytoplasm being composed of a homogeneous, pale, or acidophilic substance.

4. *Scavenger Cells.*—Occasionally large phagocytic mononuclear macrophages which have ingested numerous nuclear remnants (Fig. 76) may be mistaken for unusual giant cell forms.

5. *Capillary Cross Section.*—Another structure which may be confused with abnormal giant cell types is the specially constructed blood capillary of the lymphatic tissue of the lymph nodes and the appendix when it is viewed in cross section (Fig. 77). These capillaries are lined by a single layer of cuboidal endothelial cells instead of those of the usual pavement cell type. Lymphocytes are frequently found migrating through the walls of these structures, which thus serve as a mode of egress for the lymphocytes in addition to the efferent ducts. When, as is frequently the case, the lumen is found to contain red corpuscles as well as lymphocytes, the true nature of the structure is apparent at a glance. In inflammatory reactions, however, the cuboidal cells may become swollen so as to occlude the lumen, making identification difficult unless serial sections are available; in these the identity of the structures is readily apparent (Maximow and Bloom<sup>18</sup>).

#### COMMENT

The structure of the giant cells of the blood-forming organs in both physiologic and pathologic processes has been presented in atlas form. Attention of the hematologist is directed to variations of the giant mesenchymal structures of the hematopoietic organs in conditions not ordinarily of prime hematologic interest, for the reason that such affections are of the same stromal site or origin as are many of the blood dyscrasias. The use of dry-fixed smears or impression

preparations of the blood-forming organs in conjunction with tissue sections affords another source of information regarding structural characteristics of many giant cell types.

It is hoped that the collection of photomicrographic representations of the many giant cell types within the scope of a single article will provide an opportunity for ready comparison of both related and unrelated giant cell forms and will serve as an aid in the determination of the nature and degree of such relationships or differences.

## REFERENCES

1. Haythorn, S. R.: Multinucleated Giant Cells. *Arch. Path.* 7: 651-713, 1929.
2. Slider, E. M., and Downey, H.: *Methods for the Study of Leucocytes*, in McClung, C. E.: *Handbook of Microscopical Technique*, ed. 2, New York, 1937, Paul B. Hoeber, Inc., pp. 324-343.
3. Kolouch, F., Jr.: The Lymphocyte in Acute Inflammation, *Am. J. Path.* 15: 413-433, 1939.
4. Howell, W. H.: Observations Upon the Occurrence, Structure, and Function of the Giant Cells of the Bone Marrow, *J. Morphol.* 4: 117-130, 1890.
5. Kölliker, A.: Sitzung am März 2. *Phys. med. Gesellsch. im Würzburg*, 1872. (Reference 1.)
6. Wright, J. H.: The Origin and Nature of the Blood Plates, *Boston M. & S. J.* 154: 643-645, 1906.
7. Wright, J. H.: The Histogenesis of the Blood Platelets, *J. Morphol.* 21: 263-278, 1910.
8. Dameshek, W., and Miller, E.: The Megakaryocytes in Idiopathic Thrombocytopenic Purpura, a Form of Hypersplenism, *Blood* 1: 27-53, 1946.
9. Jürgens, R., and Graupner, H.: Darstellung eines Entwicklungssystems der Thrombocyten. Zugleich ein Beitrag über das "Blutplättchenbild" der Blutkrankheiten, *Folia haemat.* 57: 263-282, 1937.
10. Strumia, M. M.: *Hematological Tables With Brief Notes*, Bryn Mawr Hospital, Bryn Mawr, Pa., 1937.
11. Rohr, K.: Das menschliche Knochenmark, Leipzig, 1940, Georg Thieme.
12. Ferrata, A., and Negreiros-Rinaldi: Sulla genesi, sulla struttura dei megacariociti in condizioni normali e patologiche e sui loro rapporti colle piastrine del sangue circolante, *Folia med.*, Napoli 1: 815, 1915.
13. Downey, H., and Nordland, M.: Hematologic and Histologic Study of a Case of Myeloid Megakaryocytic Hepato-splenomegaly, *Folia haemat.* 62: 1-39, 1939.
14. Custer, R. P.: Ueber den Ursprung der Knochenmarksriesenzellen bei extramedullärer Knochenmarkbildung, *Virchows Arch. f. path. Anat.* 288: 212-222, 1933.
15. Downey, H.: The Origin of Blood Platelets, *Folia haemat.* 15: 25-58, 1913.
16. di Guglielmo, G.: Sul sistema delle cellule giganti midollari, *Haematologica* 6: 156-195, 1925.
17. Rosenthal, N.: Blood Platelets and Megakaryocytes, in Downey, H.: *Handbook of Hematology*, vol. 1, New York, 1938, Paul B. Hoeber, Inc., pp. 449-496.
18. Bunting, C. H.: Blood Platelet and Megalokaryocyte Reactions in the Rabbit, *J. Exper. Med.* 11: 541-552, 1909.
19. Maximow, A., and Bloom, A.: *A Textbook of Histology*, ed. 4, Philadelphia, 1943, W. B. Saunders Company.
20. Frank, E.: Die hämorrhagischen Diathesen, in Schittenhelm, A., editor: *Handbuch der Krankheiten des Blutes und der blutbildenden Organe*, vol. 2, Berlin, 1925, Julius Springer, pp. 289-472.
21. Limarzi, L. R., and Schleicher, E. M.: The Reaction of Peripheral Blood and Bone Marrow in Chronic Hemorrhage and in Essential Thrombopenic Purpura, *J. A. M. A.* 114: 12-18, 1940.
22. Paseyro, P.: Contribución de la citología en el diagnóstico de las afecciones de la sangre y de los órganos hematopoyéticos, *An. Fac. de med. Montevideo* 30: 612-848, 1945.
23. Jones, O. P.: Cytology of Pathologic Marrow Cells With Special Reference to Bone Marrow Biopsies, in Downey, H.: *Handbook of Hematology*, vol. 3, New York, 1938, Paul B. Hoeber, Inc., pp. 2043-2101.
24. Sternberg, C.: Ueber eine eigenartige unter dem Bilde der Pseudoleukämie verlaufende Tuberculose des lymphatischen Apparates, *Ztschr. f. Heilk.* 19: 21-91, 1898.
25. Reed, D.: On the Pathologic Changes in Hodgkin's Disease With Special Reference to Tuberculosis, *Rep. Johns Hopkins Hosp.* 10: 133-196, 1902.
26. Jaffe, R. H.: The Reticulo-Endothelial System, in Downey, H.: *Handbook of Hematology*, vol. 2, New York, 1938, Paul B. Hoeber, Inc., pp. 975-1271.

26. Mallory, F. B.: Cited by Haythorn.<sup>1</sup>
27. Langhans, T.: Das maligne Lymphosarkom (Pseudoleukämie), *Virchows Arch. f. path. Anat.* 54: 509, 1872.
28. Oberling, C.: Les réticulosarcomes et les réticulo-endothéliosarcomes de la moelle osseuse (sarcomes d'Ewing), *Bull. Assoc. franç. p. l'étude du cancer* 17: 259-296, 1928.
29. Warren, S., and Picena, J. P.: Reticulum Cell Sarcoma of Lymph Nodes, *Am. J. Path.* 17: 385-394, 1941.
30. Gall, E. A., and Mallory, T. B.: Malignant Lymphoma, *Am. J. Path.* 18: 381-429, 1942.
31. Foot, N. C.: *Pathology in Surgery*, Philadelphia, 1945, J. B. Lippincott Company.
32. Ewald, O.: Die leukämische Retikuloendotheliose, *Deutsches Arch. f. klin. Med.* 142: 222-228, 1923.
33. Downey, H.: Monocytic Leucemia and Leucemic Reticulo-Endotheliosis, in *Handbook of Hematology*, vol. 2, New York, 1938, Paul B. Hoeber, Inc., pp. 1273-1333.
34. Custer, R. P.: Certain Lesions of Lymph Nodes, *Army Institute of Pathology*, Washington, D. C., 1943.
35. Ewing, J.: *Neoplastic Diseases*, ed. 4, Philadelphia, 1940, W. B. Saunders Company.
36. Rustizky, J.: Untersuchungen über Knochenresorption und Riesenzellen, *Virchows Arch. f. path. Anat.* 59: 202, 1874.
37. Sabin, F. R., and Miller, F. R.: Normal Bone Marrow, in Downey, H.: *Handbook of Hematology*, vol. 3, New York, 1938, Paul B. Hoeber, Inc., pp. 1789-1838.
38. Geschickter, C. F., and Copeland, M. M.: *Osteitis Fibrosa and Giant Cell Tumors*, *Arch. Surg.* 19: 169-271, 1929.
39. Geschickter, C. F., and Copeland, M. M.: *Tumors of Bone (Including the Jaws and Joints)*, ed. 2, New York, *Am. J. Cancer*, 1936.
40. Jaffe, H. L., Lichtenstein, L., and Portis, R. B.: Giant Cell Tumor of Bone, *Arch. Path.* 30: 993-1031, 1940.
41. Piney, A., and Hamilton-Paterson, J. L.: *Sternal Puncture*, ed. 3, New York, 1946, Grune & Stratton, Inc.
42. Oberling, C., and Raileanu, C.: Nouvelles recherches sur les réticulosarcomes de la moelle osseuse (Sarcomes d'Ewing), *Bull. Assoc. franç. p. l'étude du cancer* 21: 333-347, 1932.
43. Stout, A. P.: A Discussion of the Pathology and Histogenesis of Ewing's Tumor of Bone Marrow, *Am. J. Roentgenol.* 50: 334-342, 1943.
44. Michels, N. A.: The Plasma Cell, *Arch. Path.* 11: 775-793, 1931.
45. Erf, L. A., and Herbut, P. A.: Comparative Cytology of Wright's Stained Smears and Histologic Sections in Multiple Myeloma, *Am. J. Clin. Path.* 16: 1-12, 1946.
46. Fleischhacker, H., and Klima, R.: Beitrag zur Kenntnis des multiplen Myeloms, der plasmacellulären Leukämie und des plasmacellulären Granuloms. Mit besonderer Berücksichtigung der biptischen Knochenmarksuntersuchung, *Folia haemat.* 56: 5-17, 1936.
47. Herbut, P. A., and Erf, L. A.: Lipoblastic and Megakaryocytoid Multiple Myeloma, *Am. J. Clin. Path.* 16: 13-21, 1946.
48. Lubarsch, O.: Zur Myelomfrage, *Virchows Arch. f. path. Anat.* 184: 213, 1906.
49. Moss, W. T., and Ackerman, L. V.: Plasma Cell Leukemia, *Blood* 1: 396-406, 1946.
50. Patek, A. J., and Castle, W. B.: Plasma Cell Leukemia, *Am. J. M. Sc.* 191: 788-793, 1936.
51. Jackson, H., Jr., Parker, F., Jr., and Bethea, J. N.: Studies of Diseases of the Lymphoid and Myeloid Tissues. II. Plasmacytomas and Their Relation to Multiple Myelomata, *Am. J. M. Sc.* 181: 169-180, 1931.
52. Osgood, E. E., and Hunter, W. C.: Plasma Cell Leukemia, *Folia haemat.* 52: 369-383, 1934.
53. Pick, L.: Niemann-Pick's Disease and Other Forms of So-called Xanthomatosis, *Am. J. M. Sc.* 185: 601-616, 1933.
54. Klenk, E.: Ueber die Natur der Phosphatide der Milz bei der Niemann-Pickschen Krankheit, *Ztschr. f. physiol. Chem.* 229: 151-156, 1934.
55. Bloom, W.: The Histogenesis of Essential Lipoid Histiocytosis (Niemann-Pick's disease), *Arch. Path.* 6: 827-859, 1928.
56. Rowland, R. S.: II. Constitutional Disturbances of Lipid Metabolism, in Brennemann, J.: *Practice of Pediatrics*, vol. 3, Hagerstown, Md., 1937, W. F. Prior Company, Inc., pp. 15-84.
57. Mandelbaum, F. S., and Downey, H.: Histopathology and Biology of Gaucher's Disease, *Folia haemat.* 20: 139-202, 1916.
58. Lieb, H.: Cerebrosid-speicherung bei Splenomegalie, Typus Gaucher, *Ztschr. f. physiol. Chem.* 140: 305, 1924.
59. Pinkus, F., and Pick, L.: Zur Struktur und Genese der symptomatischen Xanthome, *Deutsche med. Wchnschr.* 34: 1426-1431, 1908.

60. Syllabus of Dermal Pathology, American Academy of Dermatology and Syphilology, Army Institute of Pathology, Washington, D. C., 1944.
61. Thannhauser, S. J., and Magendantz, H.: The Different Clinical Groups of Xanthomatous Diseases; a Clinical Physiological Study of 22 Cases, *Ann. Int. Med.* 11: 1662-1746, 1938.
62. Alagna, G.: Histopathologische Veränderungen der Tonsille und der Schleimhaut der ersten Luftwege bei Masern, *Arch. f. Laryngol. u. Rhinol.* 25: 527-530, 1911.
63. Warthin, A. S.: Occurrence of Numerous Large Giant Cells in the Tonsils and Pharyngeal Mucosa in the Prodromal Stage of Measles; Report of 4 Cases, *Arch. Path.* 11: 864-874, 1931.
64. Finkeldey, W.: Ueber Riesenzellbefunde in den Gaumenmandeln, zugleich ein Beitrag zur Histopathologie der Mandelveränderungen im Maserninkubationsstadium, *Virchows Arch. f. path. Anat.* 281: 323-329, 1931.
65. Finkeldey, W.: Riesenzellbefunde bei akuter Wurmfortsatzentzündung. Ein Beitrag zur Histopathologie der Veränderungen des Wurmfortsatzes im Maserninkubationsstadium, *Virchows Arch. f. path. Anat.* 284: 518-525, 1932.
66. Hathaway, B. M.: Generalized Dissemination of Giant Cells in Lymphoid Tissue in the Prodromal Stage of Measles, *Arch. Path.* 19: 819-824, 1935.
67. Mulligan, R. M.: Genesis of the Multinucleated Giant Cells in the Lymphatic Tissue of the Appendix in Measles, *Arch. Path.* 37: 61-67, 1944.
68. Corbett, E. U.: The Visceral Lesions in Measles, *Am. J. Path.* 21: 905-919, 1945.
69. Langhans, T.: Ueber Riesenzellen mit wandständigen Kernen in Tuberkeln und die fibröse Form des Tuberkels, *Virchows Arch. f. path. Anat.* 42: 383, 1868.
70. Michelson, H. E.: The Occurrence of Tuberculoid Reactions in the Inguinal Glands of Early Syphilis, *Arch. Dermat. & Syph.* 19: 66-76, 1929.
71. Forbus, W. D.: *Reaction to Injury*, Baltimore, 1943, Williams & Wilkins Company.
72. Wolbach, S. B.: A New Type of Cell Inclusion, Not Parasitic, Associated With Disseminated Granulomatous Lesions, *J. Med. Research* 19: 243-257, 1911.
73. Ash, J. E., and Spitz, S.: *Pathology of Tropical Diseases*, Philadelphia, 1945, W. B. Saunders Company.
74. Meloney, H. E.: The Histopathology of Kala-azar in the Hamster, Monkey, and Man, *Am. J. Path.* 1: 147-168, 1925.
75. Schaumann, J.: Lymphogranulomatosis Benigna in the Light of Prolonged Clinical Observations and Autopsy Findings, *Brit. J. Dermat.* 48: 399-446, 1936.
76. Moore, M.: Blastomycosis, Coccidioidal Granuloma, and Paracoccidioidal Granuloma, *Arch. Dermat. & Syph.* 38: 163-190, 1938.
77. Fox, R. A., and Rosahn, P. D.: Lymph Nodes in Disseminated Lupus Erythematosus, *Am. J. Path.* 19: 73-99, 1943.

# MITOCHONDRIA AND THEIR RELATION TO THE SO-CALLED HYALOPLASM

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## INTRODUCTION

**D**URING the past fifteen years considerable morphologic evidence has been collected which indicates that genuine megaloblasts have no role in normal erythropoiesis.<sup>1, 2</sup> But megaloblasts do, however, play the major role in the pathologic erythropoiesis which occurs in bone marrow from patients with a liver principle deficiency anemia. Before arriving at these conclusions, it was necessary to study material similar to that which Doan and associates<sup>3</sup> used while formulating their theory for avian and mammalian erythropoiesis. Because of the studies of Sabin<sup>4</sup> in 1921 on vitally stainable granulation in erythroblasts, blood drawn from chick blastoderms on the second and third days of incubation was considered the best single source of cells which Doan and associates would accept unequivocally as megaloblasts. In my studies of the blood from these chick blastoderms it was considered desirable not only to study it vitally according to Sabin's method, but also to employ Doan and associates' supravital technique and to check Sugiyama's<sup>5</sup> findings with Wright's stain.

Although my observations on chick erythroblasts stained vitally with neutral red confirm those made by Sabin, observations made on Wright stained dry films did not correspond to either Sabin's or Sugiyama's description. First, the nuclei were not as empty or devoid of structure as described by Doan and associates, nor as poorly preserved as illustrated by Sugiyama. Second, the cytoplasm of these primitive red blood cells had much more structure than described by Sabin and it differed in degree from the cytoplasm described and illustrated by Sugiyama. The thing which attracted my attention was the presence in the cytoplasm of pale or yellowish areas of hyaloplasm, sometimes discrete, not unlike those described by Downey<sup>6</sup> in cells from certain cases of leukemic reticulo-endotheliosis. These observations alone did not seem to be so important until similar cells stained supravitaly with either janus green or pinacyanol were found to have a distribution of mitochondria practically identical with the areas of hyaloplasm as seen in dry fixed smears. Because of the apparent high degree of correlation between these structures seen in cells prepared by two different methods, it was tentatively concluded that the so-called hyaloplasm represented the negative images of mitochondria. Due to an interest in the placental transfer of antipernicious anemia principle<sup>7</sup> and the development of a presumptive test for it,<sup>8</sup> studies made originally on chick primitive erythroblasts were carried over to similar cells in the yolk sac of the eleven-day rat embryo. Normally these yellowish areas or flecks in the basophilic cytoplasm are distributed so as to fall into three main categories,

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namely, diffuse, perinuclear, and a juxtannuclear clump. The latter was of particular interest since the administration of liver extract, desiccated hogs' stomach, or synthetic folic acid produced an increase in the number of cells having a juxtannuclear clump of the cytoplasmic pale areas in dry fixed material and of the mitochondria in the supravital material.

The present paper describes results which represent the termination of an extended period of observation and the beginning of an analysis of the cytoplasm with various techniques applicable to the material studied.

#### METHODS

Twenty dozen chicken eggs were incubated for periods ranging from two to nine days. Blood was drawn from the blastoderms and studied according to the techniques described by Sabin<sup>4</sup> and Sugiyama.<sup>5</sup> Pinacyanol<sup>9</sup> was usually the dye use for supravital technique, but janus green and neutral red were also used, especially for the studies on chick blood cells.<sup>10</sup> Throughout the course of investigating several related problems, 4,187 eleven-day rat embryos were obtained from seventy-nine control and 308 test pregnant Wistar rats. Blood from the yolk sac<sup>11</sup> was studied by means of various techniques. In supravital studies, amniotic fluid was withdrawn by means of a fine capillary pipette before delivering the rat yolk sac. A drop of this was placed on a specially prepared slide and yolk sac swished around in it. Then the preparation was covered and sealed with petroleum jelly. Although blood cells from embryos whose mothers received various antianemic substances were studied as the occasion arose, only observations on normal yolk sac blood are considered in this article. Dry films of blood from 1,657 embryos were stained with the May-Grünwald-Giemsa combination of Pappenheim within six hours after they were made. Permanent mitochondrial preparations were made from dry films of both embryonic chick and rat blood cells. The former were fixed and stained according to Schridde's<sup>12</sup> original technique, with the exception that differentiation was carried out in 1 per cent methyl green rather than picric acid alcohol. Practically all of the mitochondrial preparations of embryonic rat blood cells were made by adapting the standard Bensley-Cowdry technique<sup>13</sup> to this material. Cover slip preparations were dried rapidly and placed immediately in either Regaud's formalin bichromate or acetic osmic bichromate for eighteen to twenty-four hours. They were washed in tap water for two or three minutes followed by a brief bath in distilled water. The use of permanganate and oxalic acid was unnecessary. The cover slip preparations with the fixed film directed upwards were placed on slides to facilitate handling while staining with aniline acid fuchsin. Heat was applied for ten seconds and the preparation allowed to cool. This was repeated two or three times. Either methyl green or Wright's stain applied for ten to fifteen seconds was used for differentiation and counterstaining. Dehydration was accomplished by rapidly passing the cover slips through two changes of absolute alcohol followed by xylene. The distribution and localization of lipid material was studied with the aid of sudan black, which was introduced by Lison<sup>14</sup> and later employed by Baker<sup>15</sup> for the study of Golgi element. Freshly dried cover slip preparations were placed in Baker's formol-calcium fixative for eighteen to twenty-four hours. After washing in distilled water for one or two minutes, they were passed through 50 and 70 per cent alcohol and stained in a saturated solution of sudan black in 70 per cent alcohol for ten minutes. Cover slips were placed with the fixed smears facing downward in the sudan black in order to prevent dye particles from collecting on cells. Small glass rods or a metal paper clip were used to elevate the cover slips from the bottom of the staining dish and thereby permit an even distribution of stain. Excessive amounts of the dye were extracted by passing the preparation through three changes of 50 per cent alcohol for exactly ten seconds in each change of alcohol. They were then washed in distilled water for about two minutes and mounted in glycerol.<sup>16</sup> No counter stain was used. The presence of vitamin A was detected by using the Carr-Price reagent of 30 per cent antimony trichloride in chloroform.<sup>17</sup> Cover slip preparations, which had been kept in the dark, were inverted on a clean slide and two parallel



edges were sealed with petroleum jelly. This aided in channeling the reagent and prevented the cover slip from moving when the immersion lens was focused. The preparation was then examined under low power for a suitable field of primitive erythroblasts which when found was examined with the oil immersion lens. The reagent was permitted to flow beneath the cover slip by capillary attraction. The presence of vitamin A was indicated by a fugitive light greenish blue color reaction.

Phase microscopy<sup>18</sup> has been a great aid in this problem. The optical differentiation of cytoplasmic structures having small differences in their optical path was made possible by selecting an oil immersion lens with the proper diffraction plate. Bright contrast was obtained with a 1.8 mm. objective,  $0.2A + 0.25 \lambda$ , and dark contrast with 1.8 mm. objectives,  $1.0B-0.25 \lambda$  and  $2.3B-0.33 \lambda$ . Unstained dry films were inverted on a drop or two of either Baker's formol-calcium or 10 per cent formalin and ringed with petroleum jelly. Such preparations have remained in good condition for two months. After studying cells with phase microscopy, it was then possible to mark them for restudy with a bright field microscope following Wright's stain or Baker's formol-sudan black. Oil and petroleum jelly were removed by camel's hair brush moistened with xylene. Cover slip preparations were washed briefly in distilled water, dried, and stained with Wright's stain. They were again washed briefly in distilled water, dried in air, cleared in xylene, and mounted in clarite. Both bright- and dark-contrast phase microscopy were used with sudan black preparations. Although dark contrast was used in combination with the supravital technique, preference was given to studies of unstained dry films with bright contrast phase microscopy. The combination of dark contrast phase microscopy and sudan black brought out more detail than either technique alone. All of these studies were done with a Spencer research microscope.

#### OBSERVATIONS

##### *A. Embryonic Chick Blood Cells.—*

1. *Dry Films of Blood From Chick Blastoderms Incubated Two Days:* This material should be of interest to all students of the megaloblast-normoblast problem since, according to Doan and associates<sup>3</sup> and Sabin,<sup>4</sup> practically all of the cells present are megaloblasts, in the sense of their definition. However, no matter how intriguing this phase of the problem may be, it must not detract from our present interest in the cytoplasm. Some of these cells are illustrated on Plate I and designated as primitive erythroblasts according to Maximow.<sup>19</sup> Regardless of whether Wright's stain or May-Grünwald-Giemsa is used, one of the most striking characteristics is the very basophilic cytoplasm which agrees with the observations made by Doan and associates<sup>3</sup> and Dawson<sup>20</sup> but not with those of Sugiyama<sup>5</sup> who described it as having a reddish tinge. Another constant characteristic of this basophilic cytoplasm is that it is not uniform and homogeneous, contrary to Doan and associates<sup>3</sup> universal description for such cells. It is mottled with light areas of what Pappenheim<sup>21</sup> called hyaloplasm (paraplasm). These areas are neither uniform in their size and shape, nor their distribution. For example, in cell 1, *a*, Plate I, there are faint filamentous light areas, while in cell 1, *b*, there is a definite juxtannuclear light area in addition to others scattered throughout the cytoplasm. The juxtannuclear area of cell 1, *b*, may be what some authors have called the region of the centrosphere, which according to Dawson<sup>20</sup> is indistinct but according to Sugiyama<sup>5</sup> appears as a clear red spot. At no time were large perfectly clear spots lacking substance found as described and illustrated by Sugiyama. Such an area in his Fig. 4 was in all probability an artifact.

The nuclei of these primitive erythroblasts have one or two large well defined nucleoli. They may be round or irregular. The nuclear chromatin and its characteristic pattern, however, are something which escaped the attention of Sabin,<sup>4</sup> Doan and associates,<sup>3</sup> Sugiyama,<sup>5</sup> and Dawson.<sup>20</sup> It is very definitely not pale, scanty, and obscure as these authors have reported. To the contrary, the nuclear pattern is quite distinctive and is not identical with that of any cell normally found in the bone marrow. The chromatin is arranged in more or less angular particles varying in size. They are not as uniform in size and distribution as those of genuine megaloblasts of pernicious anemia bone marrow during relapse.<sup>1, 2, 22</sup> There is a sharp demarcation between the chromatin and parachromatin (basil- and oxychromatin). Although the parachromatin is conspicuous in some cells, it never exceeds the chromatin in amount. Some of the chromatin particles have delicate strands connecting them. There is usually a marked tendency for it to concentrate in the vicinity of the nucleoli. Sugiyama's<sup>5</sup> Fig. 8 does a great injustice to these cells.

2. *Supravital Preparations of Blood From Chick Blastoderms Incubated Two Days:* Either janus green or pinacyanol was used for these studies. In general, the mitochondria were either rod or coccoid forms like those described by Doan and associates<sup>3</sup> for the rabbit and Sugiyama<sup>5</sup> for the chick. Their number varied from 28 to 57. In addition to these there were also occasional filamentous forms which did not stain as intensely as the other forms. In no instance were the long filaments the only type of mitochondria present as reported by Dawson.<sup>20</sup> In many cells the mitochondria were scattered throughout the cytoplasm but in others they had a very definite perinuclear and juxtannuclear arrangement not unlike the clear areas shown in cell 1, b, Plate I. Their position seemed to be relatively stable since they would maintain their perinuclear distribution even though the cells were flattened and made to move by gentle pressure on the cover slip. They really gave the impression of clinging to the nucleus as Cunningham and Tompkins<sup>23</sup> have described for other cells. In dead or damaged cells, the mitochondria would move and oscillate, indicating a change in the cytoplasm from a gel to sol state.

After preparations were studied with a bright-field microscope, they were then examined with dark-field illumination; the deep purple or dark blue mitochondria stained with pinacyanol appeared orange and the green mitochondria stained with janus green appeared reddish. However, the most important finding was that not all of the mitochondria had been stained selectively. The number of unstained refractile mitochondria varied from 1 to 6 per cell. No counts were made to determine the relative proportion of cells with all of their mitochondria stained. It was first thought that the concentration of the dye, its distribution on the slide, or the duration of exposure might have something to do with the nonstaining of some mitochondria, but subsequent studies on the rat primitive erythroblast have lead to the conclusion that mitochondria within a single cell differ in their composition.

3. *Permanent Mitochondrial Preparations of Blood From Chick Blastoderms Incubated Two Days:* After the previously stated observations were made it

was tentatively concluded that the light areas, as seen in dry films of primitive erythroblasts stained with the usual hematologic dyes, represented negative images of mitochondria. Investigations of this problem along more critical and analytical lines had to be postponed for about seven years. However, these observations have now been verified and another method of study was introduced. It was felt that since all mitochondria do not stain supravitaly, and this reaction is probably due to the presence of a proteolytic enzyme,<sup>24</sup> one of the more specific techniques should be applied to this material. Only moderate success was obtained when Schridde's modification of Altmann's technique was used.<sup>12</sup> The cells were rounded up and the distribution of mitochondria was not readily made out. Then too, a great many cells were lost from the cover slips. Both of these difficulties were overcome when the first part of Freifeld's technique<sup>25</sup> was used, namely, the rapid drying of films or imprints before immersion in the fixing fluid. It is understood that this means drying in air at room temperature until the films appear dry. After examining these preparations and noting the clear-cut mitochondrial distribution as that in cells 2, a,

#### PLATE I

All cells were photographed at the same magnification of 1850 diameters. Cell X is a red blood cell from the maternal placental circulation to be used as a comparison of size. Nuclei of most cells are out of focus in order to bring out cytoplasmic detail. With the exception of cells 1 and 2, all cells are primitive erythroblasts from the yolk sacs of eleven-day rat embryos.

1, Blood from chick blastoderm after two and one-half days incubation. Wright's stain. Cytoplasm is quite basophilic. Light areas of the so-called hyaloplasm vary in shape and distribution. Cell 1, a, has filamentous clear areas; cell 1, b, a juxtanuclear clump; and cell 1, c, a diffuse distribution.

2, Blood from chick blastoderm after two and three-quarter days incubation. Modified Schridde's mitochondrial technique using methyl green for differentiation and counterstaining. Most of the mitochondria tend to be cocci shaped but some short plump rods and filaments were present. In cell 2, a, the mitochondria have a diffuse distribution. Faint filaments are present in the lower portion of the cytoplasm. Cell 2, b, has a perinuclear distribution of mitochondria.

3, May-Grünwald-Giemsa stain. Cytoplasm is deeply basophilic. This cell is of particular interest because there are filamentous areas of the so-called hyaloplasm. Some of these areas are quite curved and may appear horseshoe shaped like that shown in the lower left portion of the cytoplasm.

4, May-Grünwald-Giemsa stain. Cytoplasm is quite basophilic with a more or less diffuse arrangement of yellowish areas of the so-called spherical area in the upper left portion of the cytoplasm. See text for discussion of its probable nature.

5, May-Grünwald-Giemsa stain. Most of the cytoplasm is very basophilic and fairly homogeneous. Note that the light areas of so-called hyaloplasm have a perinuclear arrangement. The small, dark rodlike structure at the upper margin of the cell is a piece of dust.

6, May-Grünwald-Giemsa stain. The nonmitotic cell 6, a, has a juxtanuclear clump of the so-called hyaloplasm. Some authors would describe this as a Hof in the region of the cytocentrum. Careful focusing of the areas of cells 5 and 6, a, are not homogeneous but contain many closely packed, yellowish or pale areas. Cell 6, b, is in prophase of mitosis and has a deeply stained, diffuse distribution of yellowish areas.

7, Bensley-Cowdry aniline acid fuchsin technique applied to dry smear. Most of the mitochondria are cocci shaped but there are some short rods. Note their diffuse distribution.

8, Bensley-Cowdry aniline acid fuchsin technique applied to dry smear. Morphology of mitochondria is the same as in cell 7, but the distribution is quite different. There is a good correlation between perinuclear clear areas as shown in cell 5 and the perinuclear arrangement of mitochondria.

9, Bensley-Cowdry aniline acid fuchsin technique applied to dry smear. Morphology of mitochondria is the same as in cell 7, but the distribution is different from either of the previous cells. There is a juxtanuclear clump of mitochondria, there are still some clear areas in the cytoplasm. Note the correlation between this type of mitochondrial juxtanuclear position of the clear area in cell 6.

10, Baker's formol-sudan black technique. The mitochondria appear as gray or grayish black cocci and short plump rods. Some filamentous forms may also be seen, but they for the most part do not take on as much sudan black as the other forms. This cell has a diffuse arrangement of mitochondria.

11, Baker's formol-sudan black technique. The mitochondria appear as described for cell 10, but here they have a perinuclear distribution with some slightly out of focus superimposed on the nucleus.

12, Baker's formol-sudan black technique. Morphology of mitochondria is the same as in cell 10, but the distribution is that of a juxtanuclear clump. Faint filaments are present in the lower portion of the cytoplasm and they have varying degrees of curvature. The two black spheres to the left of the nucleus belong to the Golgi element.



x

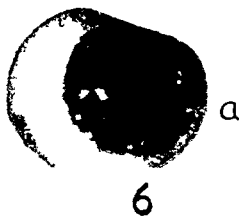
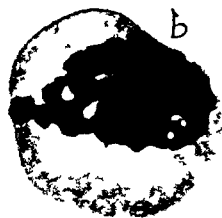


Plate I  
or legend, see opposite page.)

and 2, b, Plate I, one wonders why so little use has been made of this technique in recent years. Studies of these preparations showed a good correlation between the distribution of mitochondria and the clear areas in the cytoplasm. In addition, the modified Schridde technique stained many more filamentous mitochondria than were ever seen with either dark-field illumination or the supravital technique. This very likely is due to the fact that the action of acid fuchsin is dependent upon the lipoidal nature of mitochondria,<sup>26</sup> hence this technique would bring out mitochondria with low refractive indices not seen with dark-field illumination and those without proteolytic enzymes not stained supravitaly. The advantage of this technique is that dry films may be stained specifically for mitochondria and compared with dry films stained with the standard hematologic dyes.

### *B. Embryonic Rat Blood Cells.—*

1. *Dry Films of Blood From Yolk Sac of Eleven-Day Rat Embryo:* As indicated in the Introduction, these studies were transferred to primitive erythroblasts of the rat embryo because of the availability of material. This was advantageous because these cells are larger and have more cytoplasm. Careful examination revealed many bizarre arrangements of the so-called hyaloplasm. Cell 3 is of interest because of curved areas which, in one instance, appeared horseshoe-shaped. Areas which have a similar appearance have been described and illustrated by Sundberg and Downey<sup>27</sup> in reticular lymphocytes as representing cracks in the basophilic spongioplasm filled with clear hyaloplasm. This quite naturally implies an absence of definite structure, if we adhere to the accepted usage of the term hyaloplasm.<sup>28</sup> In all probability some of these light areas in other cells have been called trophospongium or canaliculi.<sup>29</sup> In a fair percentage of the cells examined, these areas in the cytoplasm had a very definite yellowish tinge. This perhaps would have been ignored, if it had not been recalled that Bensley<sup>30</sup> described extracted liver mitochondria as having a yellowish appearance. In spite of subsequent findings which indicated that a carotenoid pigment is present, it is still not known whether or not this coloration can be attributed to the stain or to the natural state of these cytoplasmic areas. Right or wrong, it fortified the evidence favoring the mitochondrial nature of the so-called hyaloplasmic areas and led to other investigations the results of which justified the original assumption.

Early in the course of these investigations it was thought that perhaps one way to solve this problem was to determine the incidences of various configurations of the so-called hyaloplasmic areas as seen in dry films stained with May-Grünwald-Giemsa and to correlate them with the incidences of known mitochondrial configurations. Primitive erythroblasts may be divided into three groups according to the distribution of the light areas. In the first group the cells have a more or less diffuse arrangement as illustrated by cell 4, Plate I. Such cells constitute about 60 per cent of all erythroblasts. In the second group the cells have a greater concentration of the light areas around the nucleus, forming a perinuclear Hof. In general, cytoplasm between this perinuclear clear area and the cell margin is more homogeneous than that in the first type. Compare cells 4 and 5, Plate I. The third group is made up of cells

having a fairly well-defined accumulation of clear areas more or less to one side of the nucleus, a juxtannuclear clump as shown in cell 6. In some instances this juxtannuclear light area would be separated from the nucleus by a band of basophilic cytoplasm and in others it would be in contact with the nuclear membrane. Careful examination of these juxtannuclear light areas, as well as perinuclear areas, revealed that they are composed of many closely packed yellowish or pale cocci-shaped and short plump rodlike areas. Cells of groups two and three constitute about 30 and 10 per cent, respectively, of all primitive erythroblasts. Additional illustrations of these cells may be found in articles dealing primarily with nuclear structure<sup>1, 31</sup> and in a recent one concerning cell potentialities.<sup>32</sup>

2. *Supravital Preparations of Blood From Yolk Sac of Eleven-Day Rat Embryo:* In general, the observations made on the chick cells stained with janus green or pinacyanol apply equally well for rat embryonic blood so that they need not be repeated here. However, it is important to describe the staining reaction of embryonic rat cells to neutral red. Granules or vacuoles (6 to 18) of variable size usually appeared in that region of the cell referred to as the centrosphere which in most instances also represented the area with the greatest concentration of mitochondria. All granules or vacuoles were not limited to this area because many were found around the nucleus, as described by Sugiyama<sup>5</sup> for the chick. At the present time it is impossible to state whether or not all of the structures stainable with neutral red are identical in nature. However, there are some of these which enlarge greatly with time, while the others remain more or less the same size. Unfortunately Baker's<sup>15</sup> method of using neutral red, which prevents an increase in their size, was not tried. Occasionally it was impossible to decide whether or not a few mitochondria were also stained with neutral red, especially in the case of the cocci-shaped mitochondria. Practically all of the neutral red vacuoles were colored uniformly, but in rare instances they had a chromophilic periphery and a chromophobic center. It has not been determined whether or not these would eventually develop into the first type. Cell 4 has a single clear round area in the upper left portion of the cell. This is not unlike some of the neutral red vacuoles with respect to size and position. Even though the cells in the supravital preparations were not flattened to the extent that they were in dry films, the distribution of the mitochondria corresponded to the distribution of the light areas previously described. In addition to this, there was ample evidence to indicate that some of the light areas, or so-called hyaloplasm, were due to structures which stained with neutral red in the living. In view of the recent articles by Baker<sup>15</sup> and Worley,<sup>33</sup> the latter represent part of the Golgi element.

3. *Permanent Mitochondrial Preparations of Blood From Eleven-Day Rat Embryo:* All of the evidence thus far has indicated that the so-called hyaloplasmic areas, for the most part but not exclusively, represented negative images of mitochondria. The neutral red portion of the Golgi element contributed a very small part. If the centrioles contribute anything to the formation of these areas, it must indeed be very small, since they have not been demonstrated with certainty in any of the previous or subsequent studies. Quite

early in these investigations it was deemed necessary to correlate the distribution of mitochondria in dry films with that of the light areas demonstrable by routine hematologic stains. This quantitative approach to the problem was later abandoned in favor of a more direct one, namely, phase microscopy, which will be discussed in section B, 6.

Qualitative studies revealed that, in general, mitochondria of primitive erythroblasts had three types of distribution similar to the arrangement of the light areas described in sections A, 1 and B, 1. Compare cells 4, 5, and 6 with 7, 8, 9 of Plate I. For no reason other than that of convenience, Regaud's fixative was used more frequently than acetic osmic bichromate. In cells fixed with the former it was a fairly constant occurrence for the mitochondria to be thicker. This is in agreement with Baker's<sup>15</sup> statement that potassium bichromate causes them to round up and swell. On the other hand, the acetic osmic bichromate seemed superior for the fixation of filamentous mitochondria. Cells 8 and 9 are of particular interest because in addition to the perinuclear and juxtannuclear distribution of mitochondria, quite a few are scattered throughout the cytoplasm. As a rule more mitochondria were stained with this technique than with the supravital technique. Although much criticism has been leveled against the use of dry films for cytologic studies, especially of the nucleus, it was interesting to note that mitochondria behaved as discrete structures resisting mechanical force. The evidence for this was that they were seen as fuchsinophilic bodies in the neighborhood of damaged or smashed cells. Lazarow<sup>34</sup> has reported that free mitochondria from the guinea pig liver may at times retain their exact intracellular shape.

The correlation which has been described is nothing new in hematology. Forty years ago Schridde<sup>35</sup> maintained that the perinuclear clear Hof of lymphoblasts, as seen in sections stained with azure II-eosin or methyl green-pyronin, was nothing more than the negative image of accumulations of Altmann-Schridde granules (mitochondria in present-day terminology). In a very critical review of this subject, Klein<sup>36</sup> discredited Schridde's view by stating that the mitochondria were not located in the perinuclear clear zone but more peripherad. He further maintained that the perinuclear zone was an artifact. This and other polemics of that period centering around the unitarian and dualistic theories for hematopoiesis apparently caused hematologists to forget Schridde's original observation. Although the present study does not involve lymphoblasts, it seems as though both Schridde and Klein were correct to a certain extent, if a distribution of mitochondria like that in primitive erythroblasts obtains in lymphoid cells.

4. *Formol Sudan Black Preparations of Blood From Yolk Sac of Eleven-Day Rat Embryo:* If all of the previous observations were correct, then it should be possible to apply certain histochemical techniques in order further to analyze the true nature of the so-called hyaloplasmic areas. It has been known for some time that mitochondria contain lipoid (in general sense). Bensley<sup>30</sup> reported that mitochondria from guinea pig liver contain about 35 per cent total lipoid and Goerner<sup>37</sup> found that they ranged from 27.0 to 32.4 per cent in the rabbit liver. It has been rather paradoxical to know that in spite of this

high lipid content, mitochondria did not stain with sudan III or IV.<sup>30, 38</sup> This has been attributed to the lipid being inaccessible to sudan in a lipoprotein complex, and Bensley<sup>30</sup> has suggested that these negative results were due to the fact that the fat of the mitochondria was dispersed in ultramicroscopic form. In the past, one way to unmask these lipoids was to use the rather tedious procedure of Ciaccio outlined by Bowen,<sup>29</sup> but sudan black readily accomplishes this physically rather than chemically. Lison<sup>14</sup> reported the superiority of sudan black over all other similar dyes for demonstrating total fat histochemically. This is due to the fact that sudan black has a great affinity for all lipoids and readily dissolves in them. Perhaps equally important is the fact that blue blacks and black are more discernible than the various oranges, reds, etc. Sudan black also has another important advantage which has been pointed out by Baker;<sup>15</sup> namely, that it does not have a tendency to form connecting bridges between the structure in which it is deposited and another. It was my good fortune to have Mr. E. R. Hayes, who has been studying the histochemistry of the adrenal cortex, introduce the use of sudan black in our laboratory.

After having studied mitochondria by means of the supravital and Bensley-Cowdry techniques and dark-field illumination, it was not difficult to identify them in formol-sudan black preparations. For the most part they appeared as gray or grayish black cocci and short plump rods. Filamentous forms varied in their color from gray to a color which was almost indistinguishable from that of the surrounding cytoplasm. Since sudan black was available to all parts of the cell, it is justifiable to conclude that mitochondria within a given cell differ in their lipid composition. In general, the distribution of the mitochondria could be divided into three main categories, namely, diffuse, perinuclear, and juxtannuclear, as illustrated in cells 10, 11, and 12 of Plate I. In addition to the mitochondria, there were a few spherical sudanophilic structures which constantly appeared black or bluish black. Two such structures are illustrated to the left of the nucleus in cell 12. In most instances they appeared as sudanophilic throughout, but occasionally some with sudanophobic cores were encountered. They ranged from zero to eight per cell and were perhaps more constantly associated with the accumulation of mitochondria than any other region. In view of Baker's<sup>15</sup> work, these structures are in all probability related to the Golgi element and will require further study. The present evidence suggests that a clear spherical area like that in cell 4, Plate I, represents the negative image of a portion of the Golgi element.

The sudan black preparations demonstrated that considerable sudanophilic material may be located above and below the nucleus as illustrated in cell 11. It is quite possible that this may explain the appearance of vacuole-like structures and rarefactions in some nuclei of leukemic cells.

From a cytochemical point of view, cells 11 and 12, Plate I, are interesting since the disposition of sudan black indicated that the perinuclear and juxtannuclear areas contain much more lipid than the surrounding cytoplasm. An attempt was made to localize protein in this area by the use of Millon's reagent.<sup>13</sup> Thus far it has been impossible to demonstrate that protein is more concentrated in one part of the cell than it is in another. These observations



are contrary to those of Schleicher,<sup>39</sup> whose conclusions drawn from stained preparations alone have been too far-reaching. He has assumed that the Hof of pronormoblasts contains more protein than the perinuclear zone either because the fluid state of the Hof permitted protein to migrate there in the process of drying or because there is normally a greater concentration in that particular region. Although the present article deals primarily with a study of embryonic rat blood cells, sufficient material has been studied to indicate that in general the same conditions obtain for human bone marrow. In all probability some of the oxyphilic homogeneous patches in the cytoplasm which Schleicher described were the negative images of mitochondria.

Too much emphasis cannot be placed on the necessity of having good dry films for cytologic and diagnostic purposes. They should be rapidly dried and stained almost immediately for the best results. Most of us have obtained poor results after staining films which have been left overnight or longer. Quite recently some direct evidence was obtained which gave a clue to the possible nature of this process, which may rightfully be considered as degenerative. In order to study the stability of cytoplasmic structure in dry films, preparations were permitted to remain unfixed for four, six, and eight days, either in room air or in a desiccator. They were subsequently divided into three groups and stained by one of the following methods: May-Grünwald-Giemsa, Bensley-Cowdry, or formol-sudan black. There was certainly a loss of cytoplasmic detail as reported by Schleicher.<sup>39</sup> There was also a tendency for mitochondria to become less distinct. Fuchsinophilic and sudanophilic material appeared in portions of the cytoplasm not usually possessing these properties. The interpretation of sudan black preparations was that additional lipoid material had been made available for the dye. Perhaps this was by the breaking down of lipoprotein complexes which Lison<sup>40</sup> discusses under the heading of lipophanerosis. There may also be some spreading of lipoid material from the mitochondria to adjacent cytoplasmic areas. As to whether or not these processes are solely responsible for the alterations in the tinctorial qualities of cells stained with Romanowsky dyes, or a parallel phenomenon, cannot be stated at this time. In general, these changes were not as great in cells which were kept in the desiccator as they were in cells which have remained in room air.

Hematologists are beginning to find sudan black useful for studies other than those previously reported. Baillif and Kimbrough<sup>41</sup> studied leukocyte granules in various stages of development in both normal and pathologic blood. Eosinophilic and neutrophilic granules exhibited a sudanophilia even when they were very immature. Basophil granules were entirely sudanophobic. In our laboratory, Kibler<sup>42</sup> has found that Auer rods in acute leukemia are definitely sudanophilic, a property which distinguishes them from other forms of so-called azurophilic granulation including Kurloff bodies.

*5. Reaction of Blood From Yolk Sac of Eleven-Day Rat Embryos to Antimony Trichloride:* It already has been mentioned that quite early in these investigations the yellowish tint of the so-called hyaloplasm suggested a possible relationship of these areas to mitochondria. After this was established to a fair degree of certainly the most obvious thing to do was to deter-

mine whether or not primitive erythroblasts had carotenoid pigment localized within their mitochondria. Joyet-Lavergne<sup>43</sup> and Bourne<sup>35</sup> have applied the Carr-Price<sup>17</sup> reagent of antimony trichloride in chloroform to a wide variety of plant and animal tissues. Due to the fact that vitamin A produces an intense blue reaction with this reagent and carotene produces a greenish blue color, these authors were unable to determine exactly which of these carotenoids was present or whether both were. Goerner,<sup>37</sup> however, using chemical methods which permitted a more exact analysis of isolated mitochondria, was able to show that they contain vitamin A. In the course of studying liver, cardiac muscle, and genital glands of birds, reptiles, amphibians, and fish, Joyet-Lavergne<sup>44</sup> observed that some red cells showed traces of vitamin A. Since its absence was not the general rule, he conducted further studies on the blood of the skate. Red blood cells of the skate were grouped into three categories depending upon their reaction to the Carr-Price reagent. Cells belonging to the first group, which were the most numerous, had fine blue filaments near the nucleus with rare granules and short rods in the rest of the cytoplasm. Those of group two were richer in fine blue filaments and granules than group one. The last group contained cells which failed to give a blue reaction. In order to localize this reaction, parallel studies of blood with mitochondrial techniques were conducted and showed a distribution of these elements similar to the three categories previously listed. According to Joyet-Lavergne, this confirmed the general proposition that vitamin A is an essential constituent of mitochondria. By the use of fluorescence microscopy, Popper<sup>45</sup> concluded that the appearance of some of the fine bars or droplets in epithelial cells simulated the appearance of mitochondria.

The results of the present study on primitive erythroblasts of the rat are of interest not only because they showed that these cells give a positive reaction to the Carr-Price reagent but because it was possible to localize this reaction within a single mitochondrion. For routine determinations of the presence of carotenoid pigment the best results were obtained by examining the preparations with a 4 mm. objective. As a rule the greenish blue color reaction appeared in ten seconds and lasted for twenty to seventy seconds before it commenced to spread throughout the cytoplasm and fade. The distribution of this color reaction corresponded in general to the three types of mitochondrial arrangement. By using a half aperture on the substage condenser diaphragm or oblique lighting it was possible to localize this reaction within a single mitochondrion with the oil immersion lens. Even long filaments reacted uniformly throughout. Careful examination of a mass of mitochondria similar to those shown in cells 6, 9, and 12 would often reveal many of the individual elements. Such an exact localization was made possible only by all of the previous studies leading up to this point. Although Andersen and Levine<sup>46</sup> have described a method for differentiating vitamin A from carotene, no attempt was made to do so in the present studies. Hence, the reaction obtained in the embryonic rat cells may indicate the presence of either one or both of these substances. However, the results of Goerner's<sup>37</sup> work suggest that we were very likely dealing with vitamin A.

One of the most surprising findings in these experiments was that cover slip preparations of embryonic blood kept in the dark gave a positive reaction to antimony trichloride as long as four months after they were made. There did not seem to be an appreciable loss in the intensity of the color reaction. Bodansky<sup>47</sup> has pointed out that vitamin A in nature is quite resistant to oxidation and attributed its stability to the presence of antioxidants. Popper<sup>48</sup> observed that tissue sections kept in plasma or serum did not lose their vitamin A as rapidly as those kept in water. This he believed was due to the presence of some protective principle. Such a thing may very well explain why dry films of embryonic blood retained their vitamin A after four months, since the cells were surrounded by a thin film of dried plasma. For a discussion of the significance of the presence of vitamin A in mitochondria see Bourne.<sup>26</sup> So far it has been impossible to determine the presence of vitamin C in primitive erythroblasts.

*6. Correlation of Mitochondria With the Negative Images in the Cytoplasm by Means of Phase Microscopy:* Up to this point, both in the case of results reported by Schridde<sup>35</sup> and those previously mentioned, it has been possible to conclude that the light areas in the cytoplasm of dry films stained with Romanowsky dyes represent, for the most part, negative images of mitochondria, only by making parallel studies with several techniques and correlating the findings. Although all of the accumulated evidence has pointed in this direction, technical difficulties have made it impossible, until quite recently, to say with certainty that small groups of mitochondria or even a single mitochondrion as seen in an unstained preparation will appear as a negative image when stained with routine hematologic stains. Phase microscopy<sup>14</sup> has provided the answer to this problem.

Unstained dry films were usually mounted on a drop of Baker's formol-calcium fixative. The rationale for this was twofold. First, the mounting medium should be of such a nature that it would not only fix the cells to the cover slip but that it would also prevent the passage of lipoids into colloidal solution. Second, it should permit subsequent staining with either a routine hematologic stain or sudan black. The prime objective was to locate, by means of optical differentiation, filamentous mitochondria not necessarily associated with either perinuclear or juxtanuclear accumulations of these structures, and then determine whether or not they appeared in the stained preparations. By using this approach it was possible to decide definitely that the streaks, crevices, curved areas, and structures which suggest canaliculi in the basophilic cytoplasm are truly the negative images of mitochondria. For example, the horseshoe-shaped clear area of cell 3 represents the negative image of an underlying mitochondrion. Such a configuration is not an uncommon finding in primitive erythroblasts. However, it is important to realize that not all mitochondria will appear as negative in a dry fixed smear stained with one of the Romanowsky dyes. The extent to which they may be visualized is dependent upon how deeply they are located within the cytoplasm. As a rule the more superficial mitochondria located by bright contrast phase microscopy are the ones which will readily appear as negative images. The deeper ones which are even diffi-

cult to visualize with bright contrast are usually obscured by the overlying basophilic cytoplasm in routine preparations. If the mitochondria are grouped, as in perinuclear and juxtannuclear accumulations, then their representation as a negative image is practically assured in preparations subsequently stained with Wright's stain.

When unstained dry films are studied with bright-field microscopy considerable structure may be demonstrated in the cytoplasm, if the condenser diaphragm is closed about one half. As a matter of fact, cell 13, Plate II, contains more structure than Isaacs<sup>45</sup> allows for stained cells which he calls megaloblasts. When these cells were studied with bright- and dark-contrast phase microscopy, details previously lost in diffraction patterns were made much more distinct. Cells 14 and 15, Plate II, show short rodlike mitochondria and ringlike structures. The latter will be described more fully later. Cells 16 to 18, Plate II, are of particular interest since most of the mitochondria were distributed in the same optical plane. Attention is directed to the morphology and relative position of the mitochondria in unstained cells 16 and 17. After the same cells were dried and stained with Wright's stain, most of the filamentous forms appeared as negative images in the deeply basophilic cytoplasm (cell 18, *a*, Plate II). In addition to these, the perinuclear accumulation of mitochondria and Golgi elements shown in cells 16 and 17 also appeared as negative image areas. These are undoubtedly identical with the light areas in the cytoplasm of proerythroblasts which Naegeli<sup>25</sup> described and illustrated without knowing their true nature. A comparison of cells 16 and 17 with cells 18, *a* and 18, *b*, Plate II, should be very instructive to hematologists because of their size differences. Measurements of the mean cell and nuclear diameters shows that, after fixation and staining with Wright's stain, the nucleocytoplasmic ratios have been increased slightly. In the case of 18, *a* it changed from 60 to 67.4 per cent. This was due to the fact that alcoholic fixation caused a relatively greater shrinkage of the cytoplasm than that of the nucleus. In section B, 3 it was pointed out that mitochondria in damaged cells behaved as discrete structures resisting mechanical force. In cell 18, *a* the curved filamentous light area (mitochondrion) behaved in a similar fashion. Although its relative position with respect to the nuclear membrane and edge of the cell was altered, the morphology was undisturbed by shrinkage. In a subsequent article it will be shown that cells in dry films swell when placed in an aqueous medium and shrink with alcoholic fixation even after they have been flattened and dried. Cell 18, *b* is of interest because of the diffuse distribution of the light areas which correspond to the structures illustrated in cells 16 and 17, Plate II.

One of the most striking things revealed through phase microscopy was the high degree of organization present in the cytoplasm of primitive erythroblasts after they had been smeared and dried rapidly. A false impression is obtained if cells like those illustrated in 8, 9, 11, and 12, Plate I, are studied with one technique alone. The impression that mitochondria are more or less heaped about the nucleus was discredited through the use of phase microscopy which revealed that they have a more general distribution and that there are some forms which do not stain readily with either acid fuchsin or sudan black. This

very likely is due to the fact that the results of those techniques depend upon the fatty nature of mitochondria<sup>26</sup> which is variable, and the heat treatment may have caused some filaments to break up. In spite of all the structure revealed through phase microscopy, at no time was it possible to locate anything which even approximated what we designate as a "classical Golgi net or apparatus." Furthermore, it has also been impossible to locate in dividing cells a structure which could be called a centriole.

Much valuable information was obtained by combining the formol-sudan black technique with dark contrast phase microscopy. According to Lison<sup>49</sup> and Baker,<sup>15</sup> whatever appears blue black consists of lipoids. Hence, mitochondria must vary in their lipid content because they appear as grayish structures and some, especially the filamentous forms, must contain a minimal amount. When such forms have been suspected of being located within a certain cytoplasmic area, the use of phase microscopy has made their presence a certainty. In section B, 4 it was mentioned that the sudanophilic solid spheres and those with a sudanophobic core were probably related to the Golgi element. With the aid of dark-contrast phase microscopy it was possible to determine that in addition to these forms there are sudanophilic rings which pass around sudanophobic cores in a manner quite similar to that described by Baker.<sup>15</sup> They also resembled osmiophilic platelets of plant cells.<sup>49</sup> This finding tends to strengthen the view that the reason for not being able to locate a classical Golgi apparatus within primitive erythroblasts is probably not due to the fact that it was not preserved but rather that it does not exist as such. All of this will require further study.

7. *The Action of Ribonuclease on Basophilic Cytoplasm:* As a conclusion to all of the preceding sections which have dealt with the nature of the light areas in basophilic cytoplasm, it seems appropriate that the latter should also be analyzed in dry films. Basophilia of lymphocytes was identified by Brachet<sup>50</sup> as ribonucleoprotein by digesting sections of the spleen in a crude preparation of ribonuclease. Recently, Wislocki and Dempsey<sup>51</sup> have shown that the basophilia of definitive erythroblasts in sections of monkey's bone marrow was abolished

#### PLATE II

Cells 13 to 17 were mounted in 10 per cent formalin and photographed at a magnification of 1610 diameters. Cells 18, a, and 18, b, were mounted in clarite and photographed at a magnification of 1625 diameters.

13, Unstained cell as seen with bright-field microscope at about half aperture. Although there is considerable cytoplasmic structure in this cell, the detail is lost in diffraction patterns.

14, Bright contrast with  $0.21\lambda + 0.2\lambda$  diffraction plate. Note the rod and ring forms to the right of the nucleus. In sudan black preparations the rings appeared sudanophilic and are probably related to the Golgi element.

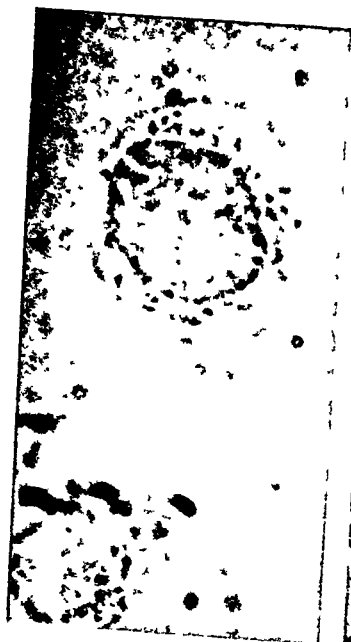
15, Dark contrast with  $2.3B - 0.33\lambda$  diffraction plate. This was focused at a slightly lower level than cell 14 in order to demonstrate structures to the left of the nucleus which appear as dark rods. Ring forms previously described may also be seen to the right of the nucleus.

16, Unstained cells in bright contrast with  $0.21\lambda + 0.2\lambda$  diffraction plate. Note the curved and straight filamentous mitochondria in the upper half of cell a. Ring forms near the nucleus are also present.

17, Same cells as 16 in dark contrast with  $2.3B - 0.33\lambda$  diffraction plate. Note the shape and relative position of the mitochondria in the upper half of cell a.

18, Same cells as in 16 and 17 after drying and staining with Wright's stain. Bright-field microscopy. The filamentous mitochondria appear as negative images in the basophilic cytoplasm. Although there has been considerable shrinkage, the morphology of the light areas is practically identical with that of the mitochondria demonstrated by phase microscopy.

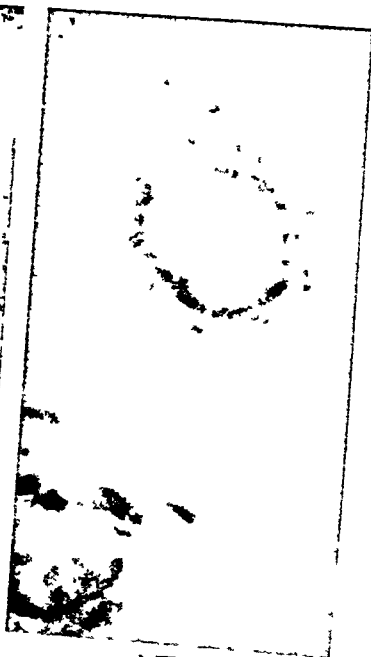
The phase photo-micrographs were furnished by the Research Division, American Optical Company, Scientific Buffalo, N. Y., and the plates were arranged by M. D. Diedrich, Director of the University of Buffalo.



13



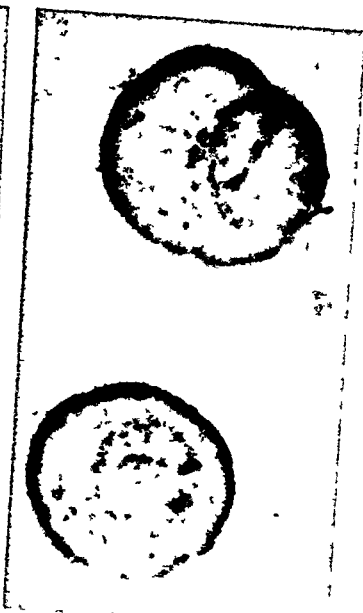
14



15



16



17



a



b

18

Plate II

(For legend, see opposite page)

after digesting them in a solution of crystalline ribonuclease. In our laboratory, cover slip preparations of dry films of embryonic blood have also been successfully treated with a similar solution of crystalline enzyme\* and the basophilic material in the cytoplasm and nucleoli has been identified as ribonucleoprotein. These preparations were fixed briefly in Zenker-formol and controls were incubated in buffer solution. Either methyl green-pyronin or Wright's stain were used as counterstains. Cytoplasm of digested cells stains quite acidophilically with the latter. At the present it is impossible to state how much of this reaction may be attributed to hemoglobin which has been uncovered.

#### DISCUSSION

It is impossible in the space allotted to discuss adequately all of the ramifications of a problem which was started over seven years ago as merely an investigation of the pale or light cytoplasmic areas of primitive erythroblasts. It has grown and extended well beyond the limits of pure morphology due to the development and application of newer histochemical and optical techniques. All of the evidence presented indicates that it is no longer logical to ascribe a hyaloplasmic nature to the clear areas of cytoplasm in primitive erythroblasts since, contrary to being the clear and more fluid ground substance of protoplasm, they actually represent the negative images of definite formed bodies. The use of the term hyaloplasm has been varied. When Pappenheim<sup>21</sup> adopted the term paraplasm (hyaloplasm) he did so not only because accurate descriptive terminology was lacking in the field of morphologic hematology, but also because most cytologists were still using this term in the sense originally employed by Leydig.<sup>52</sup> To say the least, the two terms, spongioplasm and hyaloplasm, have served a useful purpose in the absence of more specific information regarding the true nature of these substances.<sup>1, 6, 25, 27</sup> However, most cytologists have forsaken this interpretation of cytoplasmic structure in favor of one which considers the hyaloplasm to be the continuous phase of a polyphasic colloidal system. It is the clear, apparently homogeneous ground substance in which formed bodies are suspended.<sup>26, 28, 53, 54</sup> When Pappenheim<sup>55</sup> published his work he continued to use the term paraplasm (hyaloplasm) in its original sense.<sup>55</sup> During this same period Schäfer<sup>52</sup> deplored the fact that certain cytologists had introduced confusion into the terminology by employing the term hyaloplasm in a different sense. In spite of the fact that most hematologists have continued to use the terms spongioplasm and hyaloplasm (paraplasm) to describe the basophilic cytotreticulum and the lighter interspongioplasmic material present in lymphoid cells, biologists abandoned this usage long ago. In all probability the hematologist continued to use these terms because he had little or no interest in the actual structure of cytoplasm beyond what knowledge was necessary for the classification of cells.

Perhaps one of the most important findings in the present investigation is that since 1880 hematologists have been making dry film preparations which

\*Crystalline ribonuclease was kindly furnished by Dr. E. A. Sharp, Department of Clinical Investigation, Parke, Davis & Company, Detroit, Mich.

preserve cytoplasmic detail with an extraordinary degree of accuracy. It seems to me that hematologists should not be castigated so much for using the dry film method of studying blood,<sup>36</sup> as for not having endeavored to find out the nature of some of the things which they have repeatedly described and perhaps argued about. To be sure, new techniques should be devised and used but not to the extent that the older ones are not developed and utilized to their optimum. Cunningham and Tompkins<sup>23</sup> pointed out that the methods of Ehrlich and Romanowsky are not capable of demonstrating certain specific data which the supravitral technique reveals. This is only partially true in the case of primitive erythroblasts from the chick and rat, for we now know that a fairly accurate estimate of the mitochondrial distribution may be obtained from a study of good dry smears alone. This reevaluation of what can be seen in dry films and its interpretation increases the usefulness of this method for studying the influence of antianemic substances transmitted across the placenta.<sup>7, 8</sup> Cell size, nuclear structure, and the general mitochondrial distribution may all be studied in a single properly prepared dry film of embryonic blood.

The use of dry films of the embryonic yolk sac was introduced by Kirschbaum<sup>11, 31</sup> primarily for the purpose of studying the nuclear structure of mammalian embryonic blood cells and comparing these with the megaloblasts of pernicious anemia. Later, this material was found suitable for studying the influence of antipernicious anemia principle on embryonic erythropoiesis.<sup>7, 8, 57, 58</sup> The present article has revealed that this material is particularly suitable for investigating the cytochemistry of a single cell. Although the latter has been limited to only a few techniques, it is hoped that additional ones will be applicable to this material and that our knowledge will be extended in this new and interesting field. The method of approach outlined in the preceding pages may very well be applied to other cell types with slight modifications necessary to suit the individual problem.

At the present time it is impossible to predict what practical applications in clinical hematology will arise from knowledge that negative images of mitochondria are demonstrable with routine hematologic stains. Perhaps it may lead to a differentiation between macronormoblasts found in the marrows of patients with hepatic cirrhosis and those in the marrows from patients with various hemolytic anemias. It will be interesting to determine whether or not vitamin A is present in the former. Finally, megaloblasts must now be restudied in light of these recent findings.

#### SUMMARY

1. Blood from the yolk sacs of eleven-day rat embryos was studied by means of various cytologic, cytochemical, and optical techniques.
2. Freshly dried films of primitive erythroblasts preserve cytoplasmic detail to an extraordinary degree.
3. Light areas in basophilic cytoplasm previously described as hyaloplasm or paraplast represent, for the most part, the negative images of underlying mitochondria.



4. Cytochemical techniques reveal that the light areas (mitochondria) are relatively rich in lipid material and contain a carotenoid pigment, probably vitamin A. Cytoplasmic basophilia is due to ribonucleoprotein.

5. Phase microscopy makes it possible to study structures in unstained blood cells which are usually hidden in routine preparations.

#### REFERENCES

1. Jones, O. P.: Cytology of Pathologic Marrow Cells, With Special Reference to Bone Marrow Biopsies, in Downey, H.: Handbook of Hematology, New York, 1938, Paul B. Hoeber, Inc., vol. 3, p 2013.
2. Jones, O. P.: Morphologic, Physiologic, Chemical, and Biologic Distinction of Megaloblasts, Arch. Path 35: 752, 1943.
3. Doan, C. A., Cunningham, R. S., and Sabin, F. R.: Experimental Studies on the Origin and Maturation of Avian and Mammalian Blood Cells, Contrib. Embryol 16: 163, 1925.
4. Sabin, F. R.: Studies on the Blood; the Vitrally Stainable Granules as a Specific Criterion for Erythroblasts and the Differentiation of the Three Strains of the White Blood Cells as Seen in the Living Chick's Yolk Sac, Bull. Johns Hopkins Hosp 32: 314, 1921.
5. Sugiyama, S.: Origin of Thrombocytes and the Different Types of Blood Cells as Seen in the Living Chick Blastoderm, Contrib. Embryol. 18: 121, 1926.
6. Downey, H.: Monocytic Leucemia and Leucemic Reticulo-Endotheliosis, in Downey, H. Handbook of Hematology, New York, 1938, Paul B. Hoeber, Inc., vol. 2, p 127.
7. Jones, O. P.: Transmission of Antianemic Principle Across the Placenta and Its Influence on Embryonic Erythropoiesis. I. Quantitative Effect of Diets Containing Ventriculin, Arch. Int. Med. 68: 476, 1941.
8. Jones, O. P.: A Possible Presumptive Test for Antipernicious Anemia Principle, Anat Rec (Suppl 3) 82: 77, 1942.
9. Hetherington, D. C.: Pimaric acid as a Supravital Mitochondrial Stain for Blood, Stain Technol. 11: 153, 1936.
10. Sabin, F. R.: Chemical Agents: Supravital Stains, in McClung, C. L.: Handbook of Microscopical Technique, New York, 1937, Paul B. Hoeber, Inc., p 117.
11. Kirschbaum, A.: Cytological Studies of Mammalian Embryonic Blood cells, Proc. Soc. Exper. Biol & Med. 35: 542, 1937.
12. Schröder, H.: Die Kornungen der Lymphocyten des Blutes, München med. Wchnschr. 52: 1233, 1905.
13. Bensley, R. R., and Bensley, S. H.: Handbook of Histological and Cytological Technique, Chicago, 1945, University of Chicago Press.
14. Liou, L.: Sur de nouveaux colorants histologiques spécifiques des lipides, Compt. rend Soc. de biol 115: 202, 1934.
15. Baker, J. R.: The Structure and Chemical Composition of the Golgi Element, Quart. J. Micr. Sc. 85: 1, 1944.
16. Zwemer, R. L.: A Method for Studying Adrenal and Other Lipoids by a Modified Gelatin Embedding and Mounting Technique, Anat Rec. 57: 41, 1933.
17. Carr, F. H., and Price, E. R.: Colour Reactions Attributed to Vitamin A, Biochem. J. 20: 497, 1926.
18. Bennett, A. H., Jupnik, H., Osterberg, H., and Richards, O. W.: Phase Microscopy, Tr. Am. Micr. Soc 65: 99, 1946.
19. Maximow, A.: Bindegewebe und blutbildende Gewebe, in v. Mollendorff, W., editor Handbuch der mikroskopischen Anatomie des Menschen, Berlin, 1927, Julius Springer, vol. 2, p 1.
20. Dawson, A. B.: Some Observations on the Primitive and Definitive Erythrocytes of the Developing Chick, Ztschr. f. Zellforsch. u. mikr. Anat. 24: 256, 1936.
21. Pappenheim, A.: Neue zytomorphologische Studien an Blutzellen mit farbanalytischen Methoden, Folia haemat 9: 572, 1910.
22. Jones, O. P.: The Importance of Megaloblasts With a Critique of Doan, Cunningham, and Sabin's Theory for Erythropoiesis, Arch. Path. To be published.
23. Cunningham, R. S., and Tompkins, E. H.: The Supravital Method of Studying Blood Cells, in Downey, H.: Handbook of Hematology, New York, 1938, Paul B. Hoeber, Inc., vol 1, p 553.
24. Marston, H. R.: The Azine and Azonium Compounds of the Proteolytic Enzymes I, Biochem J 17: 851, 1928.
25. Naegeli, O.: Blutkrankheiten und Blutdiagnostik, ed. 5, Berlin, 1931, Julius Springer.
26. Bourne, G.: Cytology and Cell Physiology, London, 1942, Oxford University Press.

27. Sundberg, R. D., and Downey, H.: Comparison of Lymphoid Cells of Bone Marrow and Lymph Nodes of Rabbits and Guinea Pigs, *Am. J. Anat.* 70: 455, 1942.
28. Wilson, E. B.: *The Cell in Development and Heredity*, New York, 1928, The Macmillan Company.
29. Bowen, R. H.: The Methods for the Demonstration of the Golgi Apparatus, *Anat. Rec.* 39: 365, 1928.
30. Bensley, R. R.: On the Fat Distribution in Mitochondria of the Guinea Pig Liver, *Anat. Rec.* 69: 341, 1937.
31. Kirschbaum, A., and Downey, H.: A Comparison of Some of the Methods Used in Studies of Hematopoietic Tissues, *Anat. Rec.* 68: 227, 1937.
32. Block, M.: An Experimental Analysis of Hematopoiesis in the Rat Yolk Sac, *Anat. Rec.* 96: 289, 1946.
33. Worley, L. G.: The Golgi Apparatus—an Interpretation of Its Structure and Significance, *Ann. New York Acad. Sc.* 47: 1, 1946.
34. Lazarow, A.: The Chemical Structure of Cytoplasm as Investigated in Professor Bensley's Laboratory During the Past Ten Years, in Hoerr, N. L.: *Frontiers in Cytochemistry*, Lancaster, Pa., 1943, Jaques Cattell Press.
35. Schridde, H.: Myeloblasten, Lymphoblasten und lymphoblastischen Plasmazellen, *Beitr. z. path. Anat. u. z. allg. Path.* 41: 223, 1907.
36. Klein, S.: Ueber die grossen einkernigen Leukozyten des Leukamieblutes, *Folia haemat.* 10: 475, 1910.
37. Goerner, A.: Effect of Dibenzanthracene on Vitamin A and Total Lipid of Mitochondria, *J. Biol. Chem.* 122: 529, 1937.
38. Bourne, G.: Mitochondria, Golgi Apparatus and Vitamins, *Australian J. Exper. Biol. & M. Sc.* 13: 239, 1935.
39. Schleicher, E. M.: Observations on the So-called "Azurophilic Granulation" of Proerythroblasts, *Anat. Rec.* 87: 355, 1943.
40. Lison, L.: *Histochimie animale. Méthodes et problèmes*, Paris, 1936, Gauthier-Villars.
41. Baillif, R. N., and Kimbrough, C.: Studies on Leucocyte Granules After Staining With Sudan Black B and May-Grünwald-Giemsa, *J. LAB. & CLIN. MED.* 32: 155, 1947.
42. Kibler, R. S.: On the Nature of Auer Rods, *Anat. Rec.* 97: 393, 1947.
43. Joyet-Lavergne, P.: Recherches sur la catalyse des oxydo-reductions dans la cellule vivante, *Protoplasma* 23: 50, 1935.
44. Joyet-Lavergne, P.: Sur la recherche de la vitamine A dans les globules rouges du sang des vertébrés, *Compt. rend. Acad. d. sc.* 201: 1219, 1935.
45. Popper, H.: Distribution of Vitamin A in Tissue as Visualized by Fluorescence Microscopy, *Physiol. Rev.* 24: 205, 1944.
46. Andersen, A. C., and Levine, V. E.: Reaction to Differentiate Vitamin A From Carotene by Means of Antimony Trichloride, *Proc. Soc. Exper. Biol. & Med.* 32: 737, 1935.
47. Bodansky, M.: *Introduction to Physiological Chemistry*, ed. 4, New York, 1938, John Wiley and Sons, Inc.
48. Isaacs, R.: The Erythrocytes, in Downey, H.: *Handbook of Hematology*, New York, 1938, Paul B. Hoeber, Inc., vol. 1, p. 1.
49. Bowen, R. H.: Studies on the Structure of Plant Protoplasm. II. The Plastidome and Pseudochondriome, *Ztschr. f. Zellforsch. u. mikr. Anat.* 9: 1, 1929.
50. Brachet, J.: La détection histochimique des acides pentosnucléiques, *Compt. rend. Soc. de biol.* 133: 88, 1940.
51. Wislocki, G. B., and Dempsey, E. W.: Observations on the Chemical Cytology of Normal Blood and Hemopoietic Tissues, *Anat. Rec.* 96: 249, 1946.
52. Schäfer, E. A.: *Text-Book of Microscopic Anatomy*, London, 1912, Longmans, Green & Co.
53. Chambers, R.: The Physical Structure of Protoplasm as Determined by Microdissection and Injection, in Cowdry, E. V.: *General Cytology*, Chicago, 1924, University of Chicago Press.
54. Hoerr, N. L.: *Frontiers in Cytochemistry*, in Cattell, J., editor: *Biological Symposia*, vol. 10, Lancaster, Pa., 1943, Jaques Cattell Press.
55. Pappenheim, A.: *Atlas der menschlichen Blutzellen*. Supplement-Band, Jena, 1912, Gustav Fischer.
56. Bloom, W.: Some Experiments on Hematopoiesis, *Proc. Inst. Med. Chicago* 15: 322, 1945.
57. Jones, O. P.: Effects of Diets Containing Desiccated Human and Hog Stomach Preparations on Embryonic Rat Erythropoiesis, *Anat.* 88: 439, 1944.
58. Jones, O. P.: Mitotic Activity of Primitive Erythroblasts Increased by Administration of Antipernicious Anemia Preparations to Pregnant Rats, *Anat. Rec.* 85: 321, 1946.

# INDUCTION OF LEUKEMIA IN EIGHT INBRED STOCKS OF MICE VARYING IN SUSCEPTIBILITY TO THE SPONTANEOUS DISEASE

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THE incidence of spontaneous leukemia in inbred mice is dependent on the genetic constitution of the strain. If a chemical or physical agent is to be considered "leukemogenic" then its administration must result in (1) an increase in the incidence of leukemia among test as compared with control mice of the same stock, (2) an accelerated onset of the disease, or (3) both effects.

Using these criteria, agents of three general types, two chemical and one physical, have been considered to be leukemogenic. The chemical agents are first carcinogenic hydrocarbons, such as carcinogenic tar,<sup>8</sup> methylcholanthrene,<sup>11, 24, 27, 32</sup> benzpyrene,<sup>24, 31</sup> and 9, 10-dimethyl-1, 2-benzanthracene,<sup>17, 26</sup> and second various estrogens.<sup>14, 15</sup> Ionizing radiations such as x, gamma, and beta rays represent the physical agents capable of inducing this effect.<sup>7, 16, 17</sup>

Included under the term "leukemia" in mice are systemic lymphatic and myelogenous leukemia and both localized and metastatic lymphosarcoma. Certain high leukemia stocks develop all these disease types spontaneously.<sup>23</sup> The induced disease has usually been lymphoid in our experience, except in those instances where the stock of mice develops myeloid leukemia spontaneously.<sup>25</sup> Morphologically and clinically these human and mouse diseases are very similar.<sup>9</sup> Response to therapeutic agents is comparable in both species.<sup>5, 6, 12, 13</sup>

Susceptibility to the induction of leukemia cannot always be correlated with susceptibility to spontaneous leukemia.<sup>8, 21, 33</sup> Furthermore, x-rays may induce leukemia in stocks which are refractory to the chemical carcinogens.<sup>21</sup> This report represents an analysis of these phenomena in eight inbred stocks of mice.

## MATERIALS AND METHODS

Control groups for each stock of mice (except C57 Black) were observed by us. The strains used for experiments were dba-subline 212, Bagg albino, CBA, C3H, Strong A, NH, C57 Black, and F. In each stock (except C57 Black) the offspring of the controls were used as test animals. All mice were fed a diet of Purina fox chow and were permitted to breed to the extent that treatment with the leukemogenic agents allowed. A total of 1,400 mice were observed; the number used for each experiment is indicated on the bar graphs (Figs. 1 to 5) and tables.

Mice were placed on experiment at 10 weeks of age. Methylcholanthrene was administered by skin-painting, the carcinogen being dissolved in benzene as a 0.25 or 0.5 per cent solution. The solution was applied with a camel's hair brush ( $\frac{1}{8}$  by  $\frac{1}{8}$  inches), a different

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area of the skin receiving the carcinogen with each painting. Animals were painted three times weekly with the weaker and twice weekly with the stronger solution. Estrogen was administered to Bagg albino and CBA mice in the form of a subcutaneous pellet of estrone\* and to C3H animals by a single subcutaneous injection of 50 gamma weekly (throughout the experiment) of estradiol dipropionate in 0.1 c.c. of sesame oil. X-rays were administered by whole body irradiation, 140 kv., 30 cm. distance, 2 mm. aluminum filter. The mice were kept in a cardboard container while receiving radiation. Roentgens in air and interval of application are indicated on the bar graphs and their legends (Figs. 1 to 5) and in the tables (Tables I to VIII).

Leukemia was diagnosed by both gross and microscopic examination. The disease was detected in most of the animals ante mortem by physical examination.

### RESULTS

*Strain dba-Subline 212* (Table I and Fig. 1).—Following only eighteen paintings of a 0.25 per cent solution of methylcholanthrene in benzene, there was an increase in the total incidence of leukemia and an acceleration in the onset of the disease (Fig. 1, *b*). If the carcinogen was applied three times weekly and this treatment was continued until the animal was sacrificed because death seemed imminent, these effects were accentuated (Fig. 1, *c*). X-rays were also leukemogenic for this strain, but not to the same degree as methylcholanthrene (Fig. 1, *d*, *e*, and *f*). In spite of the fact that in this stock mammary cancer occurs spontaneously (Table I), and its onset was accelerated by the action of methylcholanthrene, and some females probably died as a result before leukemia developed, the incidence of leukemia was consistently higher in females (Table I). This suggests that female animals of this stock may be more sensitive than males to the development of both spontaneous and induced leukemia. Other investigators have noted that in certain stocks females develop leukemia more readily than males<sup>3</sup> and the incidence may be reduced by ovariectomy,<sup>30, 34</sup> although this phenomenon is not universal.<sup>20</sup>

It may be concluded that strain dba-212 is moderately susceptible to the development of spontaneous leukemia. Upon administration of methylcholanthrene, or exposure to x-rays in appropriate doses, leukemia appeared earlier

TABLE I. STRAIN DBA—SUBLINE 212

TREATMENT	NUMBER OF MICE			NUMBER WITH LEUKEMIA			NUMBER WITH MAMMARY CANCER*		NO LEUKEMIA OR MAMMARY CANCER SKIN CANCER
	TOTAL	MALE	FE-MALE	TOTAL	MALE	FE-MALE	TOTAL	CANCER PLUS LEUKEMIA	
None	111	47	64	13	5	8	38	2	0
MC†—X18—0.25%	27	21	6	9	3	6	4	4	5
MC†—cont—0.25%	65	26	39	33	12	21	24	8	10
X-rays—200 r-X9 biweekly	29	15	14	6	1	5	0	0	0
X-rays—100 r-X11 weekly	38	28	10	13	8	5	1	0	0
X-rays—50 r-X32 weekly	24	11	13	6	2	4	2	0	0

\*Females.

†Methylcholanthrene.

\*Estrone pellets were supplied by Parke, Davis & Company, Detroit, Mich., through the courtesy of Dr. D. K. Kitchen.

in life than in untreated mice of this strain, and the incidence of the disease was increased. Susceptibility to the leukemogenic action of estrogenic hormone could not be determined because this stock does not tolerate the doses of estrogen known to be necessary for leukemogenesis in susceptible strains.

*Strain CBA* (Table II and Fig. 2).—Five out of seventy-six untreated CBA mice developed leukemia spontaneously (Fig. 2, *a*). When treated with methylcholanthrene none of thirty-four animals developed leukemia (Fig. 2, *b*).

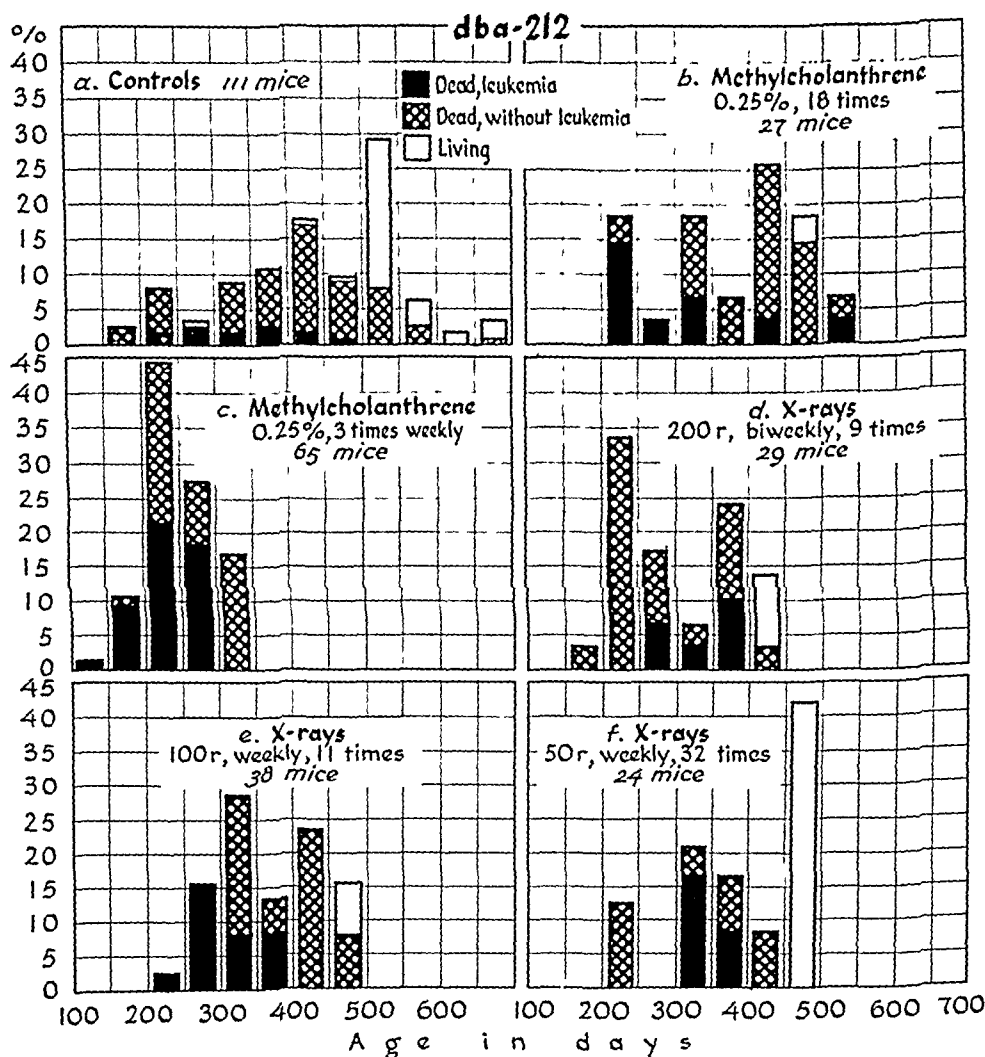
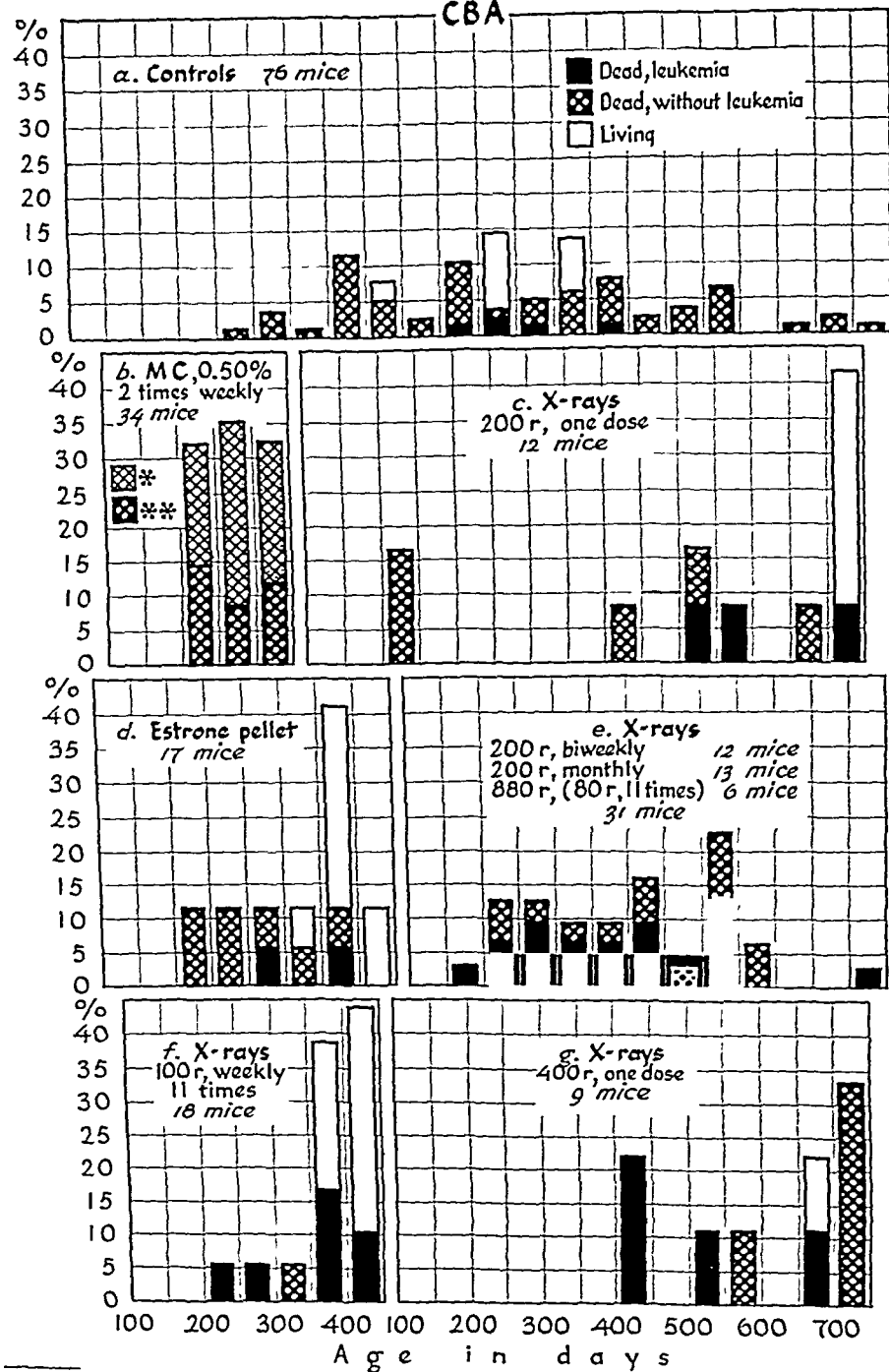


Fig. 1.—Illustrates the incidence of leukemia in controls of the dba-212 strain and in mice of this stock which were treated with methylcholanthrene or exposed to x-rays.

Because of the development of skin cancer resulting from the action of methylcholanthrene (Table II), none of these mice survived beyond 350 days of age. However, if methylcholanthrene were leukemogenic for this strain leukemia should have appeared before this age (compare Figs. 1, *c* and 2, *b*). Although

CBA



\*Indicates "dead, without leukemia"; animals received MC 0.50% plus 200 r of x-rays given on the day preceding the first skin painting.

\*\*Indicates "dead, without leukemia"; animals received only 0.50% MC.

Fig. 2.—Illustrates the incidence of leukemia in control CBA mice and in animals of the same stock which were treated with methylcholanthrene, exposed to x-rays, or treated with

CBA mice were refractory to the leukemogenic action of methyleholanthrene, x-rays proved to be a potent leukemogen (Fig. 2, *c*). A single dose of 200 or 400 r is probably above the threshold range for this strain, although such doses were not as effective as much higher doses in inducing leukemia (compare Fig. 2, *c* and *g* with *c*). Estrone induced leukemia in two out of seventeen mice (compare Fig. 2, *a* and *d*). In contrast to the dba strain leukemia was not induced more readily in female than in male animals (Table II). The development of mammary cancer was not a complicating factor in this stock.

TABLE II. STRAIN CBA

TREATMENT	NUMBER OF MICE			NUMBER WITH LEUKEMIA			NUMBER WITH SKIN CANCER
	TOTAL	MALE	FEMALE	TOTAL	MALE	FEMALE	
None	76	26	50	5	1	4	0
MC*—0.5%	34	16	18	0	0	0	31
X-rays—200 r one dose	12	1	11	3	0	3	1
X-rays—200 r-X9 biweekly, once a month, or 880†	31	19	12	18	12	6	1
X-rays—100 r-X11 weekly	18	7	11	7	4	3	0
X-rays—400 r one dose	9	6	3	4	2	2	0
Estrone pellet	17	8	9	2	1	1	0

\*Methylcholanthrene.

†80 r daily on eleven successive days with no further treatment.

TABLE III. STRAIN BALB

TREATMENT	NUMBER OF MICE			NUMBER WITH LEUKEMIA			NUMBER WITH SKIN CANCER
	TOTAL	MALE	FEMALE	TOTAL	MALE	FEMALE	
None	77	27	50	1	0	1	0
MC*—0.25 and 0.5% (Fig. 3, <i>b</i> )	50	25	25	0	0	0	40
X-rays—200 r-X9 monthly	16	5	11	10	1	9	0
Estrone pellet	16	16	0	7	7	0	0

\*Methylcholanthrene.

*Strain Balb* (Table III and Fig. 3).—Although a large number of the seventy-seven control animals are still alive, those living have reached sufficient age (Fig. 3, *a*) to serve as controls for the induction of leukemia in this stock. Only one case of leukemia has appeared among the controls. When treated with methyleholanthrene, although a large per cent of the mice developed skin cancer (Table III), these animals were of such an age (Fig. 3, *b*) that cases would have appeared in this group if methyleholanthrene were leukemogenic for this stock. When 200 r of x-rays were given monthly for nine successive months, ten out of sixteen animals developed the disease (nine of eleven females and one of five males). Estrone was almost as potent a leukemogen as x-rays from the standpoint of the total number of leukemias induced and was more active from the standpoint of latent period (compare Fig. 3, *c* and *d*). Only male mice were tested with estrone pellets, since females of this stock develop pyometra and all die within 200 days after implantation of the pellet.

Thus, Bagg albino mice were relatively refractory to the development of spontaneous leukemia, and proved to be absolutely refractory to the leukemogenic action of methyleholanthrene, but susceptible to x-rays or estrone as leukemia incitors.

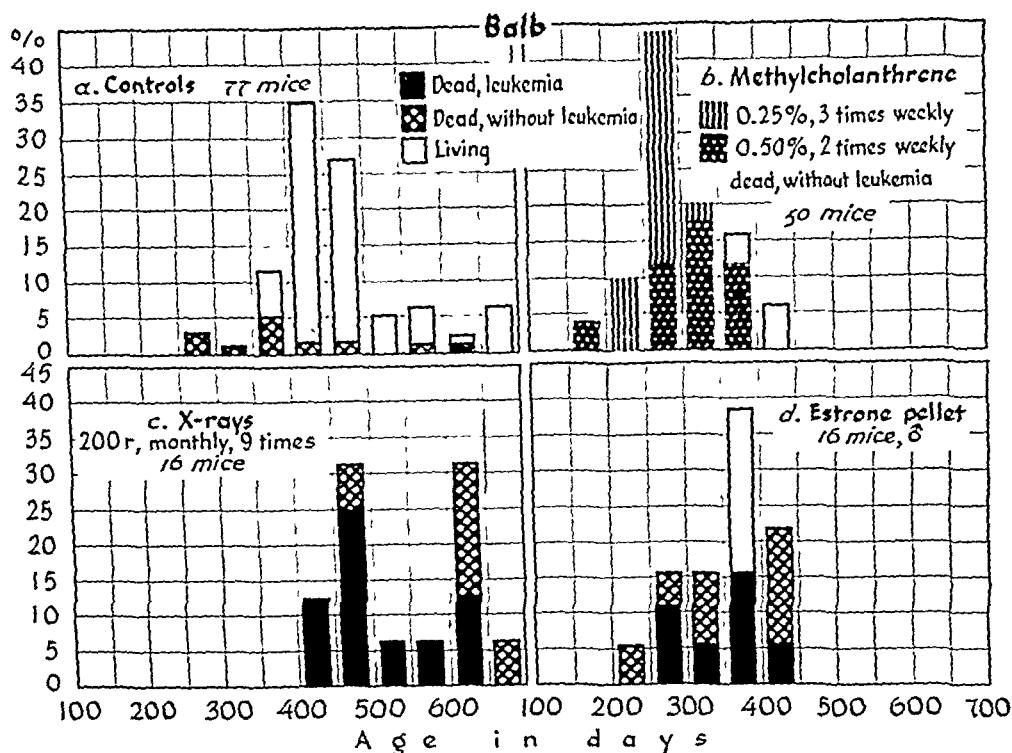


Fig. 3.—Illustrates the incidence of leukemia in untreated Balb albino mice and in groups treated with methylcholanthrene, x-rays, or estradiol dipropionate.

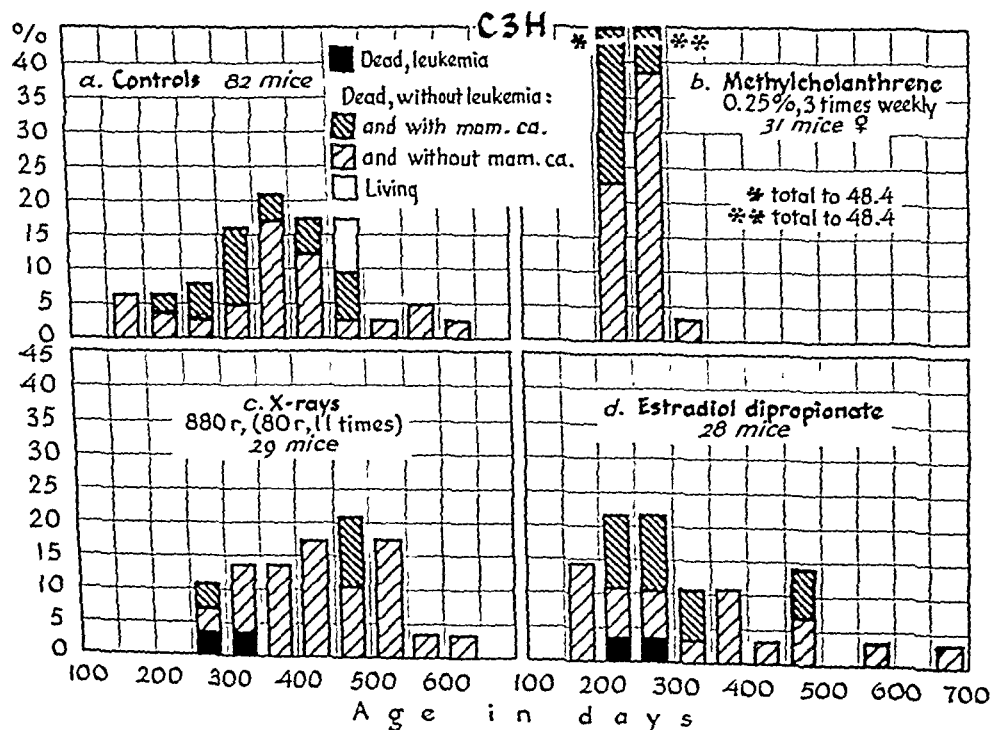


Fig. 4.—Illustrates the incidence of leukemia in control C3H mice and in mice of the same stock which were treated with methylcholanthrene, x-rays, or estradiol dipropionate.



TABLE IV. STRAIN C3H

TREATMENT	NUMBER OF MICE			NUMBER WITH LEUKEMIA			NUMBER WITH MAMMARY CANCER*		NO LEUKEMIA OR MAMMARY CANCER SKIN CANCER
	TOTAL	MALE	FE-MALE	TOTAL	MALE	FE-MALE	TOTAL	CANCER PLUS LEUKEMIA	
None	82	23	59	0	0	0	30†	0	0
MC†—cont—0.25%	31	0	31	0	0	0	11§	0	18
X-rays—880 r	29	14	15	2	1	1	5	0	0
Estradiol dipropionate	28	15	13	2	1	1	11	0	0

\*Females.

†Thirty out of thirty-nine mice with the mammary cancer "milk-agent" developed mammary cancer.

‡Twenty-three of the thirty-one females in this group lacked the mammary cancer "milk-agent."

§Methylcholanthrene.

*Strain C3H*\* (Table IV and Fig. 4).—No leukemias were observed in eighty-two control mice, nor was leukemia induced in any of thirty-one animals painted with a 0.25 per cent solution of methylcholanthrene in benzene. In a group of 29 C3H mice which received a total of 880 r of x-rays (80 r on eleven successive days), two animals developed leukemia. The same number of leukemias appeared in a group of twenty-eight animals which received 50 gamma weekly of estradiol dipropionate. In all groups a considerable number of females developed mammary cancer (Table IV). As a result of painting with methylcholanthrene skin cancer was a cause of death so that very few mice treated with the carcinogen survived beyond 300 days of age.

The C3H stock of mice is thus a low-leukemia strain which was refractory to the leukemogenic action of methylcholanthrene, but probably somewhat susceptible to the leukemia-inciting action of x-rays and estradiol dipropionate. X-rays<sup>17</sup> and estrogens<sup>14</sup> have been demonstrated by other investigators to induce leukemia in C3H mice. In a previous experiment<sup>25</sup> C3H animals were refractory to methylcholanthrene.

*Strong A Strain* (Table V and Fig. 5, a and b).—In the present experiments x-rays were the only leukemogenic agent studied. Six cases of leukemia (Fig. 5, b) appeared among fifty-eight mice which received radiation with x-rays, whereas there were no cases in the group of ninety-two controls. Thus, although leukemia did not appear in untreated mice of the Strong A strain, these mice were moderately susceptible to the leukemogenic action of x-rays. In previous studies methylcholanthrene did not induce leukemia in this stock<sup>21</sup> and estrogens were probably only very weakly leukemogenic.<sup>14</sup>

TABLE V. STRAIN STRONG A

TREATMENT	NUMBER OF MICE			NUMBER WITH LEUKEMIA			NUMBER WITH MAMMARY CANCER
	TOTAL	MALE	FEMALE	TOTAL	MALE	FEMALE	
None	92*	42	50	0	0	0	35
X-rays†	58‡	27	31	6	1	5	5

\*Eight died of liver abscess.

†200 r-X9 either biweekly or monthly, or 400 r in one dose.

‡Fourteen without leukemia died of liver abscess.

\*C3H and C57 Black mice were supplied by Dr. John J. Bittner, University of Minnesota Medical School.

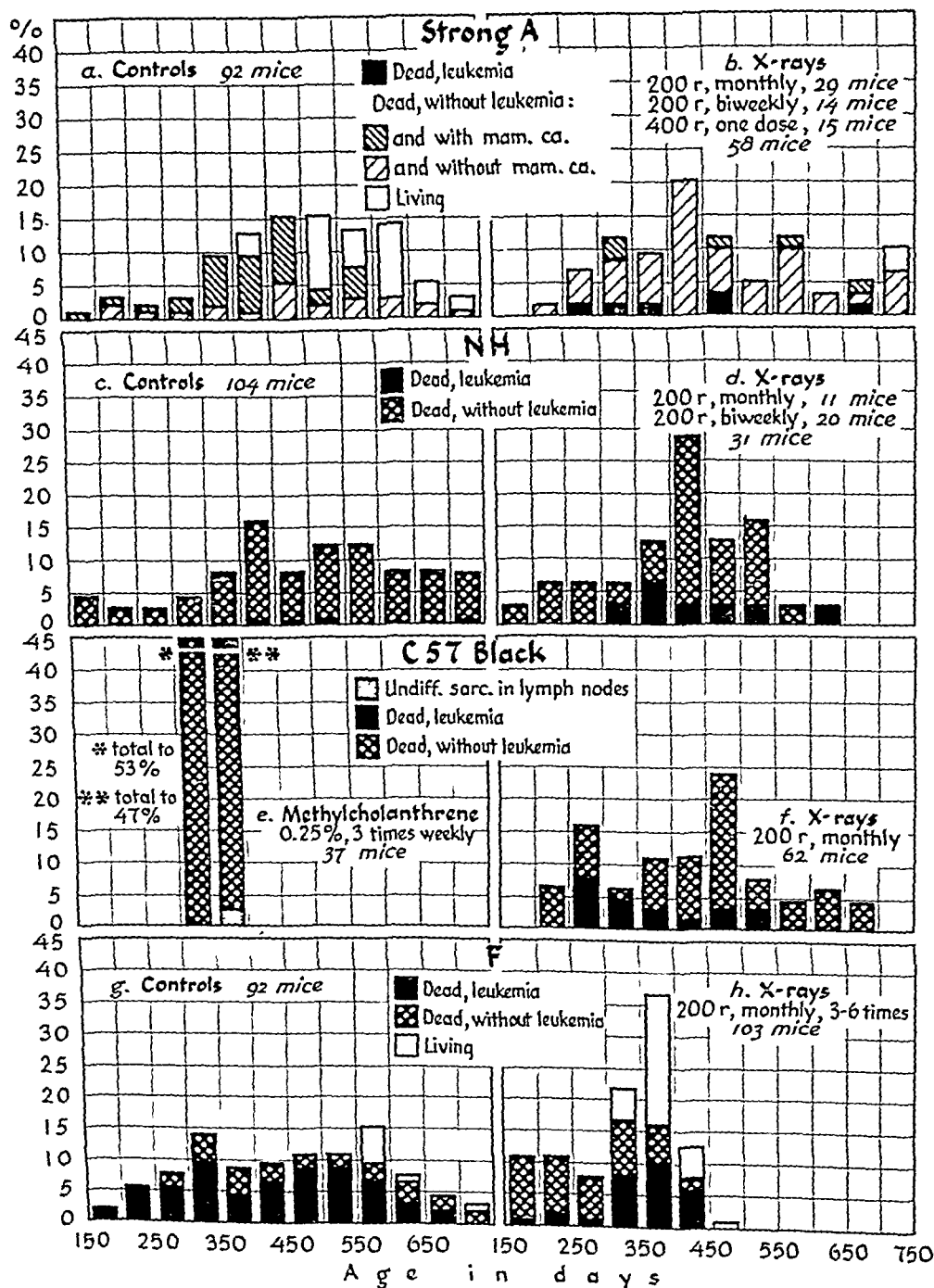


Fig. 5.—Illustrates incidence of leukemia in: a and b, Control and x-radiated Strong A mice; c and d, control and x-radiated NH mice; e and f, methylcholanthrene-treated and x-radiated C57 Black mice; g and h, control and x-radiated F mice.

*Strain NH* (Table VI and Fig. 5, *c* and *d*).—Four cases of leukemia appeared in the group of 104 controls and seven cases in thirty-one animals receiving x-rays. This stock did not tolerate estrogens well and methylcholanthrene was not tested.

*Strain C57 Black* (Table VII and Fig. 5, *c* and *f*).—These mice were refractory to induction of leukemia by methylcholanthrene, but developed leukemia following radiation with x-rays (fifteen out of sixty-two mice). Methylcholanthrene-treated mice lived to be from 300 to 400 days of age; by 400 days of age, nine of the fifteen cases of leukemia induced by x-rays had appeared. This is a low-leukemia stock which was refractory to estrogenic hormone as a leukemia incitor.<sup>14</sup> Susceptibility to the leukemogenic action of x-rays has been reported previously in this stock.<sup>16</sup>

TABLE VI. STRAIN NH

TREATMENT	NUMBER OF MICE			NUMBER WITH LEUCEMIA		
	TOTAL	MALE	FEMALE	TOTAL	MALE	FEMALE
None	104	62	42	4	2	2
X-rays—200 r-X9 monthly or biweekly	31	7	24	7	0	7

TABLE VII. STRAIN C57 BLACK

TREATMENT	NUMBER OF MICE			NUMBER WITH LEUKEMIA			NUMBER WITH SKIN CANCER
	TOTAL	MALE	FEMALE	TOTAL	MALE	FEMALE	
MC*—cont—0.25%	37	0	37	17†	0	17†	20
X-rays—200 r-X9 monthly	62	26	36	15	9	6	0

\*Methylcholanthrene.

†Undifferentiated sarcoma of lymph nodes.

TABLE VIII. STRAIN F

TREATMENT	NUMBER OF MICE			NUMBER WITH LEUKEMIA			NUMBER LIVING
	TOTAL	MALE	FEMALE	TOTAL	MALE	FEMALE	
None	92	42	50	57	26	31	7
X-rays—200 r-X3 to X6—monthly	102	55	47	29	17	12	29

*Strain F* (Table VIII and Fig. 5, *g* and *h*).—This is a high leukemia stock (Fig. 5, *g*) in which leukemia appeared precociously when the mice were skin painted with methylcholanthrene.<sup>24, 25</sup> This strain did not tolerate x-rays well in the doses given (in Fig. 5, *h* note deaths without leukemia at an early age). The relatively poor general status of these animals following radiation might account for the fact that x-rays failed to increase the incidence of leukemia during the first year of life (Fig. 5, *g* and *h*). The doses of x-rays used did not appreciably decrease the expected incidence of leukemia for this strain. Strain F mice do not tolerate estrogens in leukemogenic doses.

#### DISCUSSION

Evidently multiple agents (carcinogens, x-rays, estrogens) are capable of inducing leukemia in mice. The effectiveness of each agent depends on the

genetic constitution of the stock involved (Table IX). Although the actual mechanism of induction might be the same for each agent, specific genes probably determine whether the strain will respond to the particular stimulus. It would appear that leukemia may represent one type of neoplastic response to any one of several inciting agents.

X-rays represent the only agent which might be considered to be almost universally leukemogenic for mice. However, the degree of responsiveness to this agent varies from strain to strain. Estrogens are probably second in general effectiveness, and the carcinogenic hydrocarbons third, although when leukemogenic the last-mentioned represent the most potent leukemogen. Susceptibility to spontaneous and carcinogen-induced leukemia can be correlated (Table IX). However, some strains with a very low incidence of spontaneous leukemia developed a high incidence following treatment with carcinogenic chemicals.<sup>8, 33</sup> Benzol has been reported to induce leukemia in mice, but the experiments were not conducted under controlled conditions using inbred stocks.<sup>28, 29</sup> When dba mice were skin painted with benzene alone the incidence of leukemia did not differ from that found in controls.<sup>32</sup>

TABLE IX. PRINCIPLE OF MULTIPLE LEUKEMOGENIC FACTORS IN MICE

STRAIN OF MICE	PRECIPITATING AGENT			
	CARCINOGENIC HYDROCARBON	X-RAYS	ESTROGENS	UNKNOWN FACTORS OF SPONTANEOUS LEUKEMIA
dba-212	++++	++	Poor tolerance	±
CBA	-	++++	+	±
Balb	-	++++	++	-
C3H	-	±	±	-
A	-	+	±	-
NH	Not tested	++	Poor tolerance	±
C57	-	++	-	-
F	+++	Poor tolerance	Poor tolerance	++++
		- ?		

±, 3 to 10% leukemia.

+, 10 to 15% leukemia.

++, 15 to 35 % leukemia.

+++, 35 to 50% leukemia.

++++, More than 50% leukemia.

All three proved leukemogens also induce cancer in mice. The carcinogenic hydrocarbons, of course, induce neoplasms of many types (skin cancer, sarcomas, lung tumors). X-rays induced ovarian tumors readily<sup>10</sup> and among irradiated mice there was an increase in the incidence of miscellaneous tumors.<sup>10, 32</sup> The estrogens have been demonstrated to induce cancer of the uterine cervix<sup>1</sup> and testicular tumors of mice.<sup>16</sup> None of the other steroid hormones has been proved to be carcinogenic.

The fact that carcinogens are also leukemogens lends support to the idea that leukemia is a neoplastic disease. Transplantation studies indicate that mouse leukemia behaves in general as do the other neoplasms of this species.<sup>13, 23</sup>

In these experiments treatment with leukemia-inciting agents was begun when mice were 10 weeks of age. Susceptibility to at least two of these agents (carcinogenic hydrocarbons and x-rays) was greater if administration was

begun earlier in life.<sup>10, 31</sup> It is likely that the incidence of induced disease would be higher in most stocks if experiments were begun with younger mice. It is also possible that if younger animals were used certain stocks would prove not to be completely refractory to any agent. Under the conditions of these experiments, however, it is possible to make the comparison of leukemogenic effectiveness set forth in Table IX.

## REFERENCES

1. Allen, E., and Gardner, W. U.: Cancer of the Cervix of the Uterus in Hybrid Mice Following Long-Continued Administration of Estrogen, *Cancer Research* 1: 359, 1941.
2. Brues, A. M., and Marble, B. B.: Lymphoblastoma in Mice Following Administration of Carcinogenic Tar, *Am. J. Cancer* 37: 45, 1939.
3. Cole, R. K., and Furth, J.: Experimental Studies on the Genetics of Spontaneous Leukemia in Mice, *Cancer Research* 1: 957, 1941.
4. Engelbreth, H. J., and LeFevre, H.: Acceleration of the Development of Leukemia and Mammary Carcinomas in Mice by 9, 10-Dimethyl-1, 2-Benzanthracene, *Cancer Research* 1: 102, 1941.
5. Engstrom, R., Kirschbaum, A., and Mixer, H.: Effect of Urethane on Mouse Myelogenous Leukemia, *Science* 105: 255, 1947.
6. Flory, C. M., Furth, J., Saxton, J. A., and Reiner, L.: Chemotherapeutic Studies on Transmitted Mouse Leukemia, *Cancer Research* 3: 729, 1943.
7. Furth, J.: Studies on the Effect of Roentgen Rays on Lymphomatosis of Mice, *Am. J. Roentgenol.* 32: 377, 1934.
8. Furth, J., and Barnes, W. A.: Differences Between Malignant Blood Cells From Induced and Spontaneous Leukemias of Mice, *Cancer Research* 1: 17, 1941.
9. Furth, J., Ferris, H. W., and Reznikoff, P.: Relation of Leukemia of Animals to Leukemia of Man, *J. A. M. A.* 105: 1824, 1935.
10. Furth, J., and Furth, O.: Neoplastic Diseases Produced in Mice by General Irradiation With X-Rays, *Am. J. Cancer* 28: 54, 1936.
11. Furth, J., and Furth, O.: Monocytic Leukemia and Other Neoplastic Diseases Occurring in Mice Following Intraspinal Injections of 1:2-Benzpyrene, *Am. J. Cancer* 34: 169, 1938.
12. Furth, J., and Kabakjian, D. H.: Studies on the Effect of Continuous Exposure of Mice to Gamma Rays of Radium, *Am. J. Roentgenol.* 32: 227, 1934.
13. Furth, J., Seibold, H. R., and Rathbone, R. R.: Experimental Studies on Lymphomatosis of Mice, *Am. J. Cancer* 19: 521, 1933.
14. Gardner, W. U., Dougherty, T. F., and Williams, W. L.: Lymphoid Tumors in Mice Receiving Steroid Hormones, *Cancer Research* 4: 73, 1944.
15. Gardner, W. U., Kirschbaum, A., and Strong, L. C.: Lymphoid Tumors in Mice Receiving Estrogens, *Arch. Path.* 29: 1, 1940.
16. Henshaw, P. S.: Leukemia in Mice Following Exposure to X-Rays, *Radiology* 40: 279, 1944.
17. Henshaw, P. S.: Experimental Roentgen Injury. IV. Effects of Repeated Small Doses of X-Rays on Blood Picture, Tissue Morphology, and Life Span in Mice, *J. Nat. Cancer Inst.* 4: 513, 1944.
18. Hooker, C. W., and Pfeiffer, C. A.: The Development of Testicular Tumors in Mice of the A Strain, *Cancer Research* 2: 759, 1942.
19. Kaplan, H. S.: Observations on Radiation-Induced Lymphoid Tumors of Mice, *Cancer Research* 7: 141, 1947.
20. Kirschbaum, A.: Genetic and Certain Nongenetic Factors With Reference to Leukemia in the F strain of Mice, *Proc. Soc. Exper. Biol. & Med.* 55: 147, 1944.
21. Kirschbaum, A., and Kaplan, H. S.: Induction of Leukemia in Mice, *Science* 100: 360, 1944.
22. Kirschbaum, A., and Mixer, H.: Unpublished data.
23. Kirschbaum, A., and Strong, L. C.: Leukemia in the F Strain of Mice: Observations on Cytology, General Morphology, and Transmission, *Am. J. Cancer* 37: 400, 1939.
24. Kirschbaum, A., and Strong, L. C.: Influence of Carcinogens on the Age Incidence of Leukemia in the High Leukemia F Strain of Mice, *Cancer Research* 2: 481, 1942.
25. Kirschbaum, A., Strong, L. C., and Gardner, W. U.: Influence of Methylcholanthrene on Age Incidence of Leukemia in Several Strains of Mice, *Proc. Soc. Exper. Biol. & Med.* 45: 287, 1940.
26. Law, L. W.: The Induction of Leukemia in Mice Following Percutaneous Application of 9, 10-Dimethyl-1,2-Benzanthracene, *Cancer Research* 1: 564, 1941.

27. Lewis, M. R.: Transplantable Lymphosarcoma in Mice, *Am. J. Cancer* 34: 399, 1938.
28. Lignac, G. O. E.: Die Benzol-leukämie bei Menschen und weissen Mäusen, *Krankheitsforschung* 9: 403, 1932.
29. Lignac, G. O. E.: Die Benzol-leukämie bei Menschen und weissen Mäusen, *Klin. Wchnschr.* 12: 109, 1933.
30. McEndy, D. P., Boon, M. C., and Furth, J.: On the Role of Thymus, Spleen, and Gonads in the Development of Leukemia in a High-Leukemia Stock of Mice, *Cancer Research* 4: 377, 1944.
31. Mider, G. B., and Morton, J. J.: The Effect of Methylcholanthrene on the Latent Period of Lymphomatosis in Dilute Brown Mice, *Am. J. Cancer* 37: 355, 1939.
32. Morton, J. J., and Mider, G. B.: The Production of Lymphomatosis in Mice of Known Genetic Constitution, *Science* 87: 327, 1938.
33. Morton, J. J., and Mider, G. B.: Some Effects of Carcinogenic Agents on Mice Subject to Spontaneous Leukoses, *Cancer Research* 1: 95, 1941.
34. Murphy, J. B.: The Effects of Castration, Theelin, and Testosterone on the Incidence of Leukemia in a Rockefeller Institute Strain of Mice, *Cancer Research* 4: 601, 1944.

# EVALUATION OF BONE MARROW CONCENTRATION TECHNIQUES

## A MODIFIED METHOD FOR THE SIMULTANEOUS PREPARATION AND STAINING OF BLOOD AND BONE MARROW FILMS

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CORRELATION studies of the peripheral blood and bone marrow in diseases of the hemopoietic organs is a firmly established and universally recognized procedure. Sternal marrow aspiration allows the investigator to study the blood cells in their formative stage. This knowledge has given him a new perspective into the diagnosis and management of blood dyscrasias. Sternal aspiration is not the sole method or answer to hematologic diagnosis but it is important in the proper evaluation of abnormal blood findings. In many cases it is the key to the diagnosis. The study of an abnormal blood picture is not complete without an analysis of the sternal marrow.

The two methods of sternal marrow biopsy in general use are the trephine and aspiration. Of these two methods sternal aspiration has many advantages; thus, it is the method of choice of most investigators. The one objection to this method is that along with aspiration of bone marrow cells one must of necessity withdraw peripheral blood (sinusoidal) at the same time. This may dilute the bone marrow so greatly that when one uses fixed films he may see only a few bone marrow cells on the film. This one objection has been overcome by the use of the concentration technique.

### REVIEW OF THE METHODS USED FOR CONCENTRATING BONE MARROW CELLS

Several investigators have described methods for concentrating bone marrow cells. Reich<sup>16</sup> in 1935, by means of a specially constructed needle which must be driven into the sternum, aspirated 10 c.c. of bloody fluid. This material was mixed with 2 c.c. of 1.4 per cent sodium oxalate solution and centrifuged, and films were made of the buffy layer. Recently, Reich<sup>17</sup> has modified his original method by aspirating 4 c.c. of marrow fluid and concentrating the buffy layer a second time in a small tube. In 1937 Vogel and associates,<sup>21</sup> in some of their bone marrow studies, aspirated 2 c.c. of marrow fluid and after using one drop of 30 per cent sodium oxalate as an anticoagulant, placed some of the marrow material in a Wintrobe tube and centrifuged it for five to ten minutes. In this way a relatively accurate red cell, white cell, and platelet volume was determined. Occasionally a fat layer was seen at the top of the tube. The white cell layer was carefully removed with a Wright capillary pipette and mixed to insure uniformity, and films were made. The disad-

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vantages of the methods used by Reich and Vogel and associates are, first, that the large amount of marrow blood aspirated does not lend itself to accurate quantitative study in the hematocrit tube and, second, that the distortion of cell morphology, especially that of the nucleus, due to oxalates may be misleading. This may account for the number of unclassified cells recorded by some workers. Schleicher and Sharp<sup>18</sup> in 1937 described a method of preparing and staining bone marrow in which heparin was used as an anticoagulant. Approximately 0.5 c.c. of marrow fluid was aspirated through a specially devised sternal needle (Klima and Rosegger type<sup>9</sup>) and placed into a small paraffin-lined test tube containing a sufficient amount of heparin to prevent coagulation. The heparinized marrow fluid was pipetted into a Wintrobe hematocrit tube and centrifuged at high speed for five minutes. The white myeloid layer in the hematocrit tube, plus an equal column of red cells (peripheral blood), was mixed with a volume of plasma equal to both of the cellular layers. The mixture of bone marrow cells, red blood cells, and plasma was mixed in a paraffin-lined watch glass, and margin-free films were made with a cover slip. The films were stained with May-Grünwald-Giemsa combination. The disadvantages of this method are, first, that the small amount of marrow fluid aspirated (0.5 c.c.) does not lend itself to accurate quantitative studies in the Wintrobe hematocrit tube and, second, that the dilution of bone marrow cells by the addition of the column of red blood cells (peripheral blood) defeats the purpose of concentrating marrow blood. In the method of Schleicher and Sharp the dilution factor is directly related to the quantity of the white myeloid layer; that is, the higher the column of bone marrow cells the greater the dilution with peripheral blood (column of red cells). A. S. Gordon<sup>4</sup> in 1939 used post-mortem bone marrow material for the quantitative estimation of the cellular elements of the bone marrow and for differential studies. H. Gordon<sup>5</sup> in 1941 described a method for preparing films and sections of the marrow fluid in which dry oxalate was used to prevent clotting of the marrow fluid. Accurate cytologic identification is difficult in post-mortem material, and the changes become more pronounced the longer after death one waits to study the bone marrow cells. The effect of oxalates on cytology of bone marrow cells has been discussed elsewhere.<sup>10</sup>

#### AUTHOR'S METHOD OF OBTAINING AND CONCENTRATING BONE MARROW CELLS

The method of sternal aspiration and preparation of marrow specimens with a detailed description of the apparatus (Fig. 1) and procedure was first reported by the author<sup>10</sup> in 1939. To state it briefly: Under aseptic technique and procaine anesthesia the sternum (midline) is punctured in the second or third interspace, preferably the former site, with a specially devised 16 gauge needle.\* This needle is one devised by Klima and Rosegger modified by Poncher and the author in 1937 for a Luer syringe attachment; thus, an adapter, as required for the original needle, is not necessary. The needle is forced into the bone perpendicular to the sternum until a sudden "give" indicates that the marrow cavity has been reached (Fig. 2). The stylot is then removed. With a tight fitting, dry 5 or 10 c.c.

\*University of Illinois Sternal Needle, made by V. Muller and Company, Chicago, Ill. Although a guard is a part of the needle, from a practical point of view it is of little help in gauging the thickness of the anterior plate of the sternum. The guard serves its greatest usefulness by allowing one to obtain a firm grip of the needle while forcing it into the sternum. In obese individuals it may be necessary to remove the guard from the needle before attempting to enter the sternum.



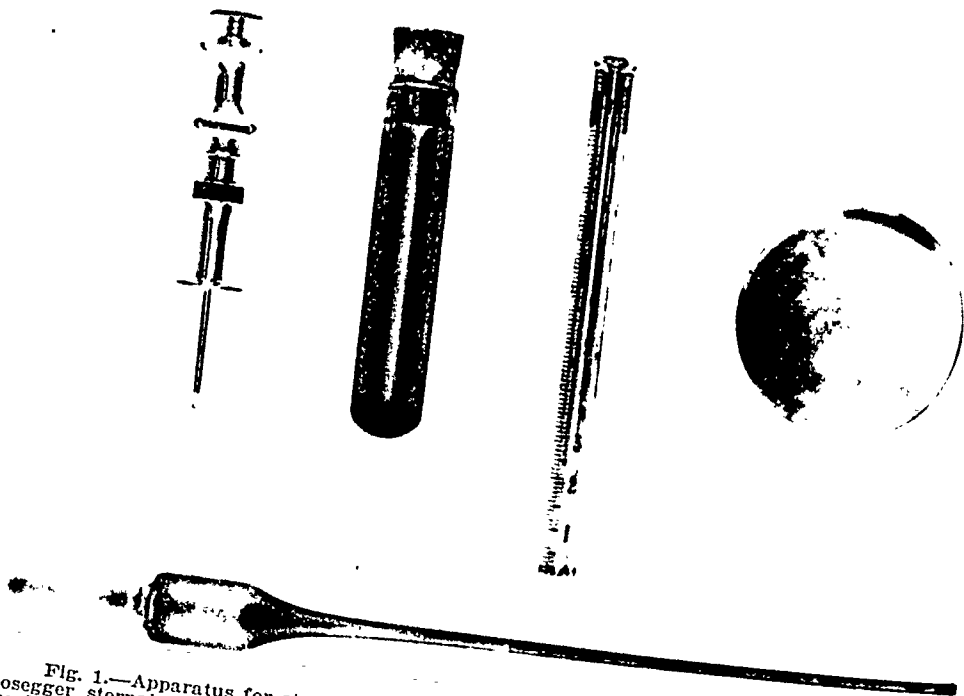
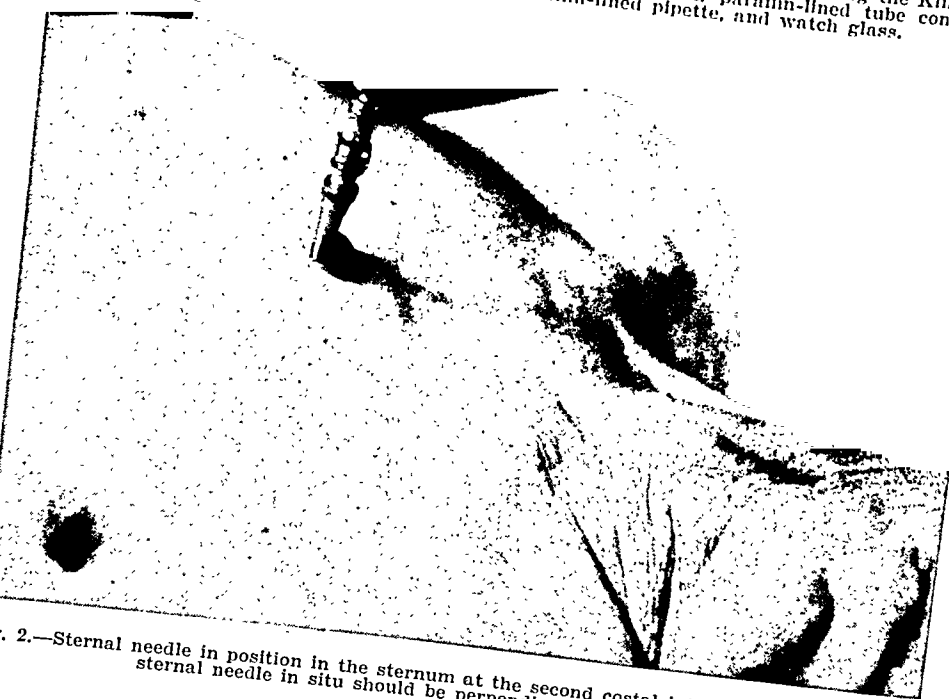


FIG. 1.—Apparatus for sternal puncture (concentration technique) showing the Klima and Rosegger sternal needle modified for Luer syringe attachment, paraffin-lined tube containing powdered heparin, Wintrobe hematocrit tube, paraffin-lined pipette, and watch glass.



2.—Sternal needle in position in the sternum at the second costal interspace. Note that the sternal needle in situ should be perpendicular to the sternum.

syringe, exactly 1 c.c. of marrow fluid is aspirated and immediately placed in a paraffin-lined tube containing a minute amount of heparin.\* The tube is gently inverted several times to insure the anticoagulant effect of the heparin. Usually, if a successful sample of bone marrow has been obtained, a more or less dense suspension of small tissue particles will be visible.<sup>10</sup> The 1 c.c. of heparinized material consisting of peripheral blood (sinusoidal), hemopoietic marrow, and fat is pipetted into a Wintrobe hematocrit tube and centrifuged for approximately five minutes at about 2,000 r.p.m. Centrifugation separates the following layers reading from top down in the tube (Fig. 3, A): (1) fat, yellow and

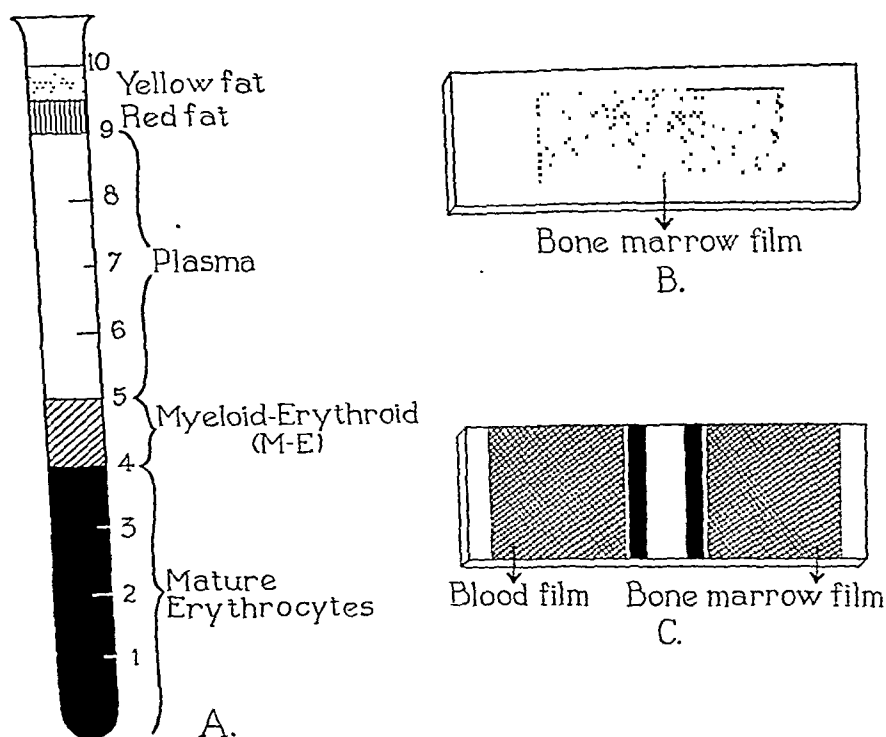


Fig. 3.—A, Hematocrit tube illustrating the composition of the several layers (volumetric) of the bone marrow material after centrifugation. B, Diagrammatic representation of the conventional bone marrow spread. The margin-free bone marrow film spread is made with an 18 by 22 mm. rectangular cover glass (same thickness as the counting chamber cover glass). C, Diagrammatic representation of a peripheral blood and bone marrow preparation with wax marking pencil lines on the inner edge of the film.

red, (2) plasma, (3) myeloid-erythroid cells; this layer also contains the megakaryocytes, and (4) erythrocytes from bone marrow sinuses. The heights of the several layers are recorded.<sup>†</sup> The fat and most of the plasma are removed separately and discarded. The myeloid-erythroid layer, together with a small amount of plasma, is pipetted off separately, transferred to a paraffin-lined watch glass, mixed, and from this material, films and cell counts are made. This method has been followed by the author and his associates in more than 3,385 practically simultaneous studies of the bone marrow and peripheral blood.<sup>11-14</sup>

\*Powdered Heparin, Lot No. 153, Hyson, Westcott, and Dunning, Baltimore, Md. This type of heparin is inexpensive and 1.0 Gm. will suffice for approximately 350 to 400 sternal punctures.

†Normal average quantitative values for adults as represented in the hematocrit tube are as follows: Fat (yellow and red), 3.2 per cent; plasma, 45 per cent; myeloid-erythroid (M-E), 6.8 per cent; and erythrocytes, 45 per cent. Before any definite figures can be accepted, a large number of cases from all age groups are needed where quantitative studies are made from marrow obtained under standardized techniques. Of these values the myeloid-erythroid is of hematologic significance.

In the original method a small drop of marrow fluid is transferred to a new and perfectly clean microslide and with an 18 by 22 mm. rectangular cover glass a margin-free film is made (Fig. 3, B). The film is stained with Wright's stain or May-Grünwald-Giemsa dyes, except that a double concentration of Giemsa stain as recommended by Ferrata<sup>3</sup> and Downey<sup>2</sup> is used. A control film that has been made from a drop of nonheparinized marrow fluid is also prepared in the same way.



Fig. 4.—The technique of making blood and bone marrow films on one microslide. A to D show the technique of making the blood film. The same technique is followed for making the bone marrow film except that the opposite edge of the microslide is used.

*Preparation of Marrow-Blood Films by Double Slide Technique.*—In order to utilize a microslide for films of both blood and bone marrow, the following technique is used: Place a small drop of concentrated marrow fluid on the near lower edge of a scrupulously clean standard 3 by 1 inches microslide approximately one-quarter of an inch from one end of the slide. With a second slide, one end of which is used as a spreader and held at an angle of 30 to 45 degrees (Fig. 4), touch the second slide to the first slide so that the drop of blood is within the angle thus formed. Draw the second slide toward the drop of blood until the end of the slide just touches the drop and the blood spreads along the acute angle by capillarity. A moment is allowed for this to occur, after which both slides are opposed and the blood "sandwiched" and spread between them. The slides are then immediately and quickly drawn apart. The first slide which contains the usable film of the bone marrow

is finally dried by air in motion.\* On the opposite end of the same slide place a drop of blood from the finger or ear and repeat the procedure as described for making the bone marrow film. This bone marrow-blood microslide preparation is now ready for staining. Before staining, a thick line by means of a marking pencil is drawn along the inner edge of both the bone marrow and blood films (Fig. 3, C).† The marking pencil line serves to prevent the stain from overflowing and prematurely staining the blood film on the opposite end of the slide. Note that the bone marrow and blood films are spread along the width of the microslide rather than the conventional length of the slide. A good bone marrow



Fig 5—Photomicrograph of the (A) peripheral blood and (B) bone marrow (megaloblastic erythropoiesis) from a patient with pernicious anemia made from a peripheral blood and bone marrow preparation as illustrated in Fig. 3, C.

\*This method was first used by Dr. Raphael Isaacs for the preparation of either blood or bone marrow films.

†When May-Grünwald-Giemsa dyes or Wright-Giemsa stains are used for staining the bone marrow-blood preparation, one can dispense with the marking pencil line. Also, when the marking pencil line that separates the blood and bone marrow films can be dispensed with and the entire preparation stained as follows: Allow the Wright's stain to act for three minutes; then dilute with distilled water as the slide will hold, mixing very thoroughly. Allow this to stand for three minutes, and then wash with distilled water and air-dry. Properly followed, the procedure will usually take care of the oversteaining of the blood.

and peripheral blood preparation should be smooth, homogeneous, and have even edges. The films of the bone marrow and peripheral blood should occupy approximately the opposite one-third of the end of the slide, as seen in Fig. 3, C. The area between the inner edges of the bone marrow and blood films, after the preparation has been stained and the marking pencil marks have been wiped away, is sufficient space for ordinary labeling.

*Staining of Bone Marrow and Peripheral Blood Preparation.*—For general routine work, Wright's stain is most satisfactory.\* The preparation is placed on a staining rack and the bone marrow film is stained first. The "square" with the bone marrow film is covered with Wright's stain and allowed to act for three minutes, this procedure serving to fix the smear. The stain is then diluted with distilled water,† as much as the lower end of the slide containing the marrow film will hold, mixing very thoroughly; the marking pencil line along the inner edge of the film prevents the stain from overflowing to the opposite side of the slide and staining the blood film prematurely. Allow this to stand approximately fifteen minutes; without washing immediately begin staining that portion of the slide with the peripheral blood film in the usual way that is, Wright's stain one and one-half minutes followed by distilled water for three minutes, mixing thoroughly. At this point the entire slide may become diluted following the overflow from both ends of the slide. The entire staining period should not exceed twenty minutes. Finally, rinse the entire slide with distilled water until the water runs, returning clear while tilting the slide. Wipe off the marking pencil marks and rinse with distilled water for a second time, place in a vertical position in a slide rack, and allow to air-dry. If this procedure is followed there will be obtained well-tinted bone marrow and peripheral blood preparations with no film or precipitated stain visible (Fig. 5).

Preparations made according to concentration technique give bone marrow patterns comparable in certain respects to those seen in bone marrow sections; thus, they are called "tissue-like" preparations. With the oil immersion objective, a differential count may be performed. This is simplified by the use of an ocular which contains a Whipple grid‡ to divide the field into segments. By moving the stage one can readily examine the peripheral blood; thus, the peripheral blood and bone marrow films are available for examination on the same microslide.

#### COMMENTS

The author's method of concentrating and preparing bone marrow has a number of advantages: (1) Myeloid-erythroid (M-E) hypoplasia and hyperplasia is relatively accurately measured; thus, quantitative changes (myeloid-erythroid volume in the hematocrit tube) as well as qualitative studies (differential counts of the bone marrow film) may be correlated. (2) The separation of the erythrocytes in the hematocrit tube, most of which are derived from the sinuses of the bone marrow, eliminates the admixture of peripheral blood that is responsible for the dilution of the bone marrow elements. (3) Fat which interferes with the proper staining of the bone marrow cells is removed by the centrifugation of the marrow fluid. It also gives a relatively accurate estima-

\*Wright's stain is prepared by placing 1 g. of certified Wright powder on the surface of 600 c.c. of chemically pure methyl alcohol in a ball mill containing one charge of pebbles. The ball mill is rotated in an electrically propelled machine overnight. The stain is stored in a 800 c.c. dark brown bottle and allowed to stand about seven days. The stain is then filtered into a 60 c.c. dispensing bottle and kept tightly corked when not in use. If a ball mill is not available, grind 0.1 Gm. of Wright powder with a few cubic centimeters of the alcohol in a mortar. Slowly add the alcohol, a few cubic centimeters at a time, until 60 c.c. of alcohol is added; then grind thoroughly. This process should stand one to two days. Filter before use.

†We have found from experience that double or triple distilled water gives better and more constant results than does a buffer solution of pH 6.4. Much of the cause of poor staining is due to the use of unclean slides or to poorly prepared films. A five gallon bottle of distilled water with an attached siphon is used for diluting Wright's stain and washing off the tinted films. The use of this water for as long as three months gives uniformly well-stained and well-tinted films.

tion of the amount of fat in the bone marrow specimen and is of value in the study of certain diseases. (4) The method lends itself to the preparation of a large collection of uniformly prepared and stained films for teaching classes in clinical pathology and hematology. The preparation of the blood film on the same microslide containing the marrow film is a practical method for the simultaneous study of hematologic material. It is an ideal method of filing both blood and bone marrow films for future reference.

One of the routine methods followed at the United States Naval Medical School<sup>15</sup> for preparing the aspirated marrow blood is to concentrate bone marrow using an ammonium-potassium oxalate mixture\* as an anticoagulant. Although this oxalate mixture causes little if any distortion of cell morphology, a control marrow film should be prepared from a drop of the marrow fluid before it is added to the anticoagulant.

A number of investigators, including Jones,<sup>5</sup> Hebbel,<sup>6</sup> Holly,<sup>7</sup> Stasney and Pizzolato,<sup>20</sup> and Schleicher,<sup>19</sup> have used the author's concentration technique for the quantitative and qualitative studies of the bone marrow in normal and pathologic conditions.

The histologic study of the bone marrow can be provided by the use of marrow particles either prior to or following heparinization and concentration of the marrow fluid. These are placed in Zenker-Formalin solution and the sections after fixation are prepared in the usual way with paraffin and stained with hematoxylin-eosin or Giemsa. Recently, Berman and Axelrod<sup>1</sup> have described a method of utilizing bone marrow aspiration material for volumetric readings, smears, imprints, and histopathologic sections. The sections are utilized in studying the topographic relationship of the bone marrow. Frequently these hemopoietic particles are too small to be of value. This is especially true in hypoplastic bone marrows. Then again detailed and accurate cell morphology and identification is not well preserved in sectioned material. Marrow blood that has been allowed to clot and treated as described for making histologic preparations is of little value for the study of bone marrow. Also, anyone whose experience with sternal aspiration has been at all intensive has observed cases in which it has been impossible to obtain bone marrow material for study, the so-called dry tap. This is observed in cases of myelosclerosis and myelofibrosis. Under such circumstances it is more appropriate to use a small trephine and remove a small plug of bone marrow. The bone marrow plug can also be used for making smears and imprint (abklatsch) preparations of the bone marrow.

Clinical hematology may derive a great deal of benefit from the study of the peripheral blood and bone marrow. However, it cannot be too strongly emphasized that data obtained by a study of all hematologic aspects of a case should never be sufficient to make a diagnosis. The clinical picture obtained by a complete history and thorough physical examination still remains the most important factor in all hematologic procedures and investigations.

\*Ammonium-potassium oxalate mixture is prepared as follows: 1.2 Gm. of ammonium oxalate and 0.5 Gm. of potassium oxalate are dissolved in 100 c.c. of distilled water. Place 0.1 c.c. of this mixture in a small test tube and evaporate to dryness. This amount of anticoagulant suffices to prevent the clotting of about 1 c.c. of aspirated bone marrow fluid.

## CONCLUSIONS

1. The advantages and disadvantages of bone marrow concentration techniques in general use for the preparation and studying of bone marrow are discussed.

2. A method is described which utilizes a microslide for the uniform preparation and staining of both peripheral blood and bone marrow films.

3. The advantages of the author's concentration technique for the study of bone marrow are discussed.

## REFERENCES

1. Berman, L., and Axelrod, A. R.: Aspiration of Sternal Bone Marrow; Technic for Obtaining Volumetric Readings, Smears, Imprints, and Histopathologic Sections, *Am. J. Clin. Path.* 17: 61-66, 1947.
2. Downey, H.: The Myeloblast, Its Occurrence Under Normal and Pathological Conditions, and Its Relations to Lymphocytes and Other Blood Cells, *Folia haemat.* 34: 65-89; 145-187, 1927.
3. Ferrata, A.: Le emopatie, Milan, 1933, Società edit. libraria.
4. Gordon, A. S.: Studies in Bone Marrow, *J. LAB. & CLIN. MED.* 24: 352-359, 1939.
5. Gordon, H.: A Method for Preparing Smears and Sections of Aspirated Sternal Marrow, *J. LAB. & CLIN. MED.* 26: 1784-1788, 1941.
6. Hebbel, R.: Bone Marrow Biopsy, *Minnesota Med.* 24: 442-449, 1941.
7. Holly, R. G.: Anemias of Pregnancy, *Staff Meet. Bull. Hosp., Univ. Minn.* 17: 281-299, 1946.
8. Jones, R. M.: Human Sternal Marrow in Hyperthyroid and Myxedematous States, *Am. J. M. Sc.* 200: 211-220, 1940.
9. Klima, R., and Rosegger, H.: Zur Methodik der diagnostischen Sternalpunktion, *Klin. Wchnschr.* 14: 541-542, 1935.
10. Limarzi, L. R.: Diagnostic Value of Sternal Marrow Aspirations, *Illinois M. J.* 75: 38-46, 1939.
11. Limarzi, L. R., Jones, R. M., Paul, J. T., and Poncher, H. G.: Sternal Marrow in Banti's Syndrome and Other Splenomegalic States; the Effect of Splenectomy, *Am. J. Clin. Path.* 13: 231-248, 1943.
12. Limarzi, L. R., Paul, J. T., and Poncher, H. G.: Blood and Bone Marrow in Infectious Mononucleosis; a Review of the Literature and a Report of Twenty-Five Cases, *J. LAB. & CLIN. MED.* 31: 1079-1100, 1946.
13. Limarzi, L. R., Poncher, H. G., and Birch, C. L.: The Bone Marrow in Hemophilia During Life; A Study of the Cellular Elements and Coagulation Time of the Marrow Blood, *J. LAB. & CLIN. MED.* 31: 777-784, 1946.
14. Limarzi, L. R.: Clinical Application of Sternal Puncture, *Proc. Inst. Med. Chicago* 16: 365-368, 1947.
15. Manual, Department of Hematology, United States Naval Medical School, National Naval Medical Center, Bethesda, Md., 1945, pp. 41-42.
16. Reich, C.: A Study of the Diagnostic Value of Sternal Puncture in Clinical Hematology, *Am. J. M. Sc.* 189: 515-523, 1935.
17. Reich, C.: A Clinical Atlas of Sternal Bone Marrow, Published by Abbott Laboratories, North Chicago, Ill., 1946, pp. 1-32.
18. Schleicher, E. M., and Sharp, E. A.: Rapid Methods for Preparing and Staining Bone Marrow, *J. LAB. & CLIN. MED.* 22: 949-951, 1937.
19. Schleicher, E. M.: The Volumetric Pattern of Aspirated Normal Human Sternal Marrow of Males 18 to 40 years, *Am. J. Clin. Path.* 14: 370-379, 1944.
20. Stasney, J., and Pizzolato, P.: Serial Bone Marrow Studies in Pernicious Anemia. I. Fluctuation in Number and Volume of Nucleated Cells, *Proc. Soc. Exper. Biol. & Med.* 51: 335-338, 1942.
21. Vogel, P., Erf, L. A., and Rosenthal, N.: Hematological Observations on Bone Marrow Obtained by Sternal Puncture, *Am. J. Clin. Path.* 7: 436-447; 498-515, 1937.

# QUANTITATIVE CYTOLOGIC STUDY OF MULTIPLE STERNAL MARROW SAMPLES TAKEN SIMULTANEOUSLY

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PREVIOUS qualitative and quantitative studies of the cytologic constituents of the bone marrow taken from widely separated portions of the marrow lead to the conclusion that there probably is a relative identity in the cellular organization of the red marrow throughout the body. Our former study was based on preparations obtained from fourteen persons after their accidental death.<sup>1</sup> The samples were obtained from three different regions shortly after death, and imprint preparations were made. In experimental work marrow samples were obtained from different portions of the marrow soon after the animal had been sacrificed.<sup>2-6</sup> With this technique we found that cytologic appraisal of any region of marrow will reveal, within the limits of error, what is the trend of the marrow changes elsewhere in the body.

Recently, several authors<sup>7-9</sup> found differences in the cellular composition of the different portions of the marrow, claiming that the marrow is not a homogenous structure. Reich and Kolb<sup>10</sup> reported a quantitative study on a series of multiple sternal marrow samples, taken simultaneously, and concluded that quantitative determinations are inaccurate.

To ascertain the basically important point whether one marrow sample would give a representative picture of the bone marrow function in the present study, we examined quantitatively and qualitatively two portions of the sternal marrow by obtaining samples from two different sites simultaneously.

## MATERIALS AND METHODS

Thirty patients with a wide variety of pathologic conditions at the Charity Hospital of Louisiana were used in this study. The marrow was aspirated through an 18 gauge spinal needle from two sites of the body of the sternum, about 2 or 3 cm. apart. Care was used to remove not more than 1 c.c. of liquid. The marrow upon removal was gently expressed through the needle into a bottle containing 2 mg. of ammonium potassium oxalate. From the material remaining in the needle, cover-slip preparations were made and rapidly dried by waving through the air; these were stained with Wright-Giemsa stain after several hours of additional drying.

From the oxalated marrow the total nucleated elements were counted employing white cell pipettes and diluting with 5 per cent acetic acid. The counts were done on the regular Levy counting chamber using 5 to 25 squares of the large central square, depending on the number of cells present. The following formula was used:

$$\text{Total nucleated elements} = \frac{\text{Number of cells counted} \times \text{dilution}}{\text{Volume of chamber}}$$

The unused marrow was placed in a Wintrobe tube and centrifugalized at 2,000 r.p.m. for five minutes and the percentage of the marrow fractions was tabulated according to the method of Limarzi.<sup>11</sup> With this technique, 5 to 8 per cent were found as normal values for

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CASE	ASPIRATION SITE	RACE	SEX	AGE	DIAGNOSIS	PERCENTAGE OF FRACTION OF MARROW				TOTAL NUCLEATED ELEMENTS PER THOUSAND	RETICULO-ENDOTHELIAL CELL	MYELOBLAST	LEUKOBLAST	PRONUCLEOCYTE	MYELOCYTE	METAMYELOCYTE	NEUTROPHILE	EOSINOPHILIC MYELOCYTE	EOSINOPHILIC METAMYELOCYTE
						FAT	PLASMA	MARROW	HEMATOCRIT										
1	1	C	M	50	Peptic ulcer	4.5	52.5	10.0	33.0	117.1	0.2	0.4	0.8	1.4	5.6	9.4	16.6	--	--
	2					4.0	55.0	8.0	33.0	68.2	0.2	0.4	0.2	0.6	2.6	9.4	17.0	--	--
2	1	C	M	50	Carcinoma of stomach	3.0	51.0	3.0	43.0	20.3	0.4	1.2	1.8	2.6	7.8	9.4	23.6	0.8	0.2
	2					1.5	53.5	2.0	43.0	17.8	0.2	0.8	1.4	3.0	10.6	12.4	23.8	0.2	0.4
3	1	C	F	53	Pernicious anemia	--	68.1	6.6	23.3	28.6	--	0.8	3.2	10.6	18.6	9.8	13.3	0.2	0.4
	2					--	69.5	4.5	26.0	22.0	0.6	2.0	4.4	15.2	16.4	6.0	7.6	0.4	0.6
4	1	C	F	71	Pernicious anemia	2.0	73.3	7.7	17.0	55.0	0.8	0.2	1.8	3.6	8.6	6.0	23.6	0.2	0.2
	2					1.4	75.8	5.7	17.1	40.8	0.8	0.6	1.2	1.6	7.8	7.2	20.0	--	--
5	1	C	F	71	Pernicious anemia*	1.1	69.7	13.5	15.7	85.5	0.4	0.2	0.6	1.2	2.6	3.2	7.4	--	--
	2					0.7	75.3	4.5	19.5	49.5	1.0	0.6	1.0	4.6	5.2	5.0	12.8	--	--
6	1	W	M	55	Pernicious anemia†	--	54.0	8.0	38.0	71.3	0.2	0.2	1.4	1.8	9.0	15.6	22.6	0.4	1.2
	2					--	56.0	6.0	38.0	46.1	0.6	0.6	0.4	4.2	4.0	12.8	28.2	--	0.2
7	1	W	M	34	Pernicious anemia‡	2.5	43.8	12.3	41.4	113.5	--	1.0	2.0	3.8	11.4	15.6	13.6	1.4	2.2
	2					2.0	50.0	5.0	43.0	47.4	0.4	0.4	1.4	5.0	8.8	13.4	18.0	1.2	1.6
8	1	W	M	50	Macrocytic anemia; received folic acid	1.5	64.5	3.0	31.0	25.1	--	0.2	0.4	1.8	5.8	10.2	28.4	0.2	0.6
	2					5.2	59.1	7.1	28.6	78.8	0.4	1.0	1.6	3.2	7.8	10.6	21.8	--	0.2
9	1	W	F	25	Bleeding peptic ulcer	3.6	56.0	7.4	33.0	72.3	--	0.4	2.2	5.2	8.8	13.6	18.4	0.2	0.2
	2					2.3	58.4	7.6	31.7	84.3	0.4	0.2	2.6	2.4	7.6	18.2	22.6	0.2	0.4
10	1	C	M	18	Sickle cell anemia	--	56.8	17.0	26.2	201.0	0.4	2.2	0.4	1.0	2.4	5.2	6.2	0.2	--
	2					--	52.0	23.0	25.0	214.0	0.4	0.3	0.8	1.8	5.0	5.8	11.2	--	0.4

\*After four days of liver treatment.

†After two weeks of liver treatment.

‡After one month of liver treatment.

bone marrow cells. We found the following distribution of the different fractions in the centrifugalized marrow in fourteen apparently healthy medical students, ten men and four women: fat, 1 to 3 per cent; plasma, 40 to 54; marrow, 3 to 11; and hematocrit, 40 to 51. The total number of nucleated elements was 43,000 to 105,000 per cubic millimeter. In the differential counts, a minimum of 500 cells was counted from each marrow sample. The damaged cells were counted also when encountered associated with well-preserved cells. The percentage distribution of all cells observed and the myeloid-erythroid ratio of each sample were computed and tabulated.

#### COMMENT

The numerical data are tabulated in Tables I to III.

In Table I, data for anemias and conditions leading to anemia are tabulated. Cases 1, 5, and 10 exhibit marked proliferation of erythroid cells with inversion of the myeloid-erythroid ratio. The values of these ratios in the two samples obtained from two sites of the sternum reveal differences but express a similar trend of changes.

	THROMBOCYTES	LYMPHOCYTES	PLASMA CELLS	PRONORMOBLAST	BASOPHILIC NORMOBLAST	POLYCHROMATIC NORMOBLAST	ORTHOCROMATIC NORMOBLAST	NORMOBLAST IN MITOSIS	MYELOID CELL IN MITOSIS	DAMAGED CELL	MEGAKARYOCYTE	IMMATURE LYMPHOCYTE	DAMAGED LYMPHOCYTE	NEUTROPHIL	PROMEGALOBLAST	BASOPHILIC MEGALOBLAST	POLYCHROMATIC MEGALOBLAST	ORTHOCROMATIC MEGALOBLAST	MYELOID CELL IN MITOSIS
1	0.2	12.0	1.2	1.0	1.4	3.2	32.4	0.8	0.2	5.8	0.8	0.6	--	0.2	--	--	--	--	--
2	0.4	12.8	1.0	1.4	3.8	12.2	24.8	0.6	--	10.2	0.4	0.6	--	0.2	--	--	--	--	--
3	--	1.6	20.4	0.4	0.2	1.2	3.4	9.8	0.4	0.2	11.0	--	0.4	--	0.6	--	--	--	--
4	--	0.4	16.2	0.4	1.0	0.6	2.4	6.2	0.2	0.3	16.4	--	--	--	0.2	--	--	--	--
5	--	--	11.8	--	2.0	0.8	0.6	8.0	0.4	0.4	13.4	--	--	--	0.6	0.2	0.4	1.0	2.0
6	0.4	--	15.0	2.2	1.6	1.0	0.8	8.4	0.2	--	9.2	--	--	--	0.6	0.2	0.6	2.2	1.2
7	--	0.2	10.6	--	3.6	4.2	3.8	3.4	1.0	0.2	16.0	0.2	0.4	--	0.4	4.2	2.4	1.8	1.4
8	--	--	16.4	0.2	2.2	4.4	4.4	6.8	0.6	--	14.2	--	--	--	--	5.4	2.4	2.2	1.0
9	--	--	7.2	--	--	2.6	42.2	4.6	0.2	0.2	25.0	0.4	--	--	--	--	--	--	--
10	0.2	--	8.2	0.2	1.2	1.6	42.2	8.2	0.8	0.4	8.2	--	0.2	--	0.2	--	--	--	--
11	0.4	--	12.6	--	0.4	2.8	1.6	21.4	0.4	--	3.0	--	1.0	--	1.0	--	--	--	--
12	0.8	0.6	19.8	0.4	0.8	0.6	1.6	10.4	0.2	--	6.0	0.2	0.2	--	1.6	--	--	--	--
13	0.4	--	9.6	--	0.8	1.4	2.6	17.6	0.2	0.2	11.0	--	1.8	--	0.6	--	--	--	--
14	0.6	0.6	10.4	0.4	0.4	1.4	2.2	12.8	0.4	0.2	14.4	--	0.3	--	0.2	--	--	--	--
15	0.8	2.2	9.8	--	0.4	1.6	6.4	16.8	0.4	--	12.6	--	--	--	0.4	--	--	--	--
16	0.2	0.8	12.4	--	0.8	2.2	8.0	16.2	1.4	--	8.0	0.2	0.4	--	0.4	--	--	--	--
17	0.2	0.2	18.8	1.0	0.2	1.0	4.8	11.6	1.0	--	6.2	--	1.6	--	1.4	--	--	--	--
18	0.2	0.2	20.6	0.2	0.4	0.6	3.4	10.6	--	--	5.8	0.2	--	--	0.8	--	--	--	--
19	--	--	11.6	0.8	3.2	8.2	12.4	31.8	1.4	--	10.4	0.4	--	--	0.6	--	--	--	--
20	--	--	11.0	0.4	1.6	5.6	11.4	36.2	1.4	0.2	4.0	--	0.2	--	--	--	--	--	--

Illustrated in Table II are cases of leukemic conditions with strikingly similar proliferation of the pathologic cells in the two samples. The case of idiopathic purpura in both samples shows relatively normal values.

In Table III are included, among others, cases with tendencies to form localized, nodular changes in the red marrow, like multiple myeloma and Boeck's sarcoidosis. The values for the myeloid-erythroid ratio, not including the pathologically proliferating cells, again reveal parallel changes in the two samples.

All the cases included were of different sex, age, and race with wide variety of pathologic conditions. Because of these great differences between the examined cases, no statistical analysis was attempted. A comparison between the cellularity of the two samples from the same sternum disclosed variations which reflected in the quantity of the different fraction of the marrow as well as in the total number of nucleated elements and in the percentage distribution of different cells.

The differences in the quantitative data may be caused by the technique of obtaining the samples. Dameshek and associates<sup>12</sup> pointed out that the tech-

ASPIRATION SITE	RACE	SEX	AGE	DIAGNOSIS	PERCENTAGE OF FRACTION OF MARROW				TOTAL NUCLEATED ELEMENTS PER THOUSAND	RETICULO-ENDOTHELIAL CELL	MYELOBLAST	LEUKOBLAST	PRONUCLEOCYTE	MYELOCYTE	METAMYELOCYTE	NEUTROPHILE	EOSINOPHILIC MYELOCYTE	EOSINOPHILIC METAMYELOCYTE	BASOPHILIC METAMYELOCYTE
					FAT	PLASMA	MARROW	HEMATOCRIT											
1	W	M	35	Leukopenia	0.6	52.3	4.7	42.4	10.6	0.6	0.2	0.6	1.6	4.2	12.4	37.6	--	0.2	0.2
2					0.7	51.2	4.6	43.5	43.6	0.6	0.6	1.0	2.8	7.2	15.2	31.6	0.2	0.2	0.2
1	C	M	53	Chronic lymphatic leukemia			Insufficient			0.2	--	--	--	0.6	0.6	1.0	--	--	--
2							Insufficient			--	--	--	--	--	0.8	0.4	--	--	--
1	C	M	40	Fever (?)	1.0	55.0	12.0	32.0	71.6	0.4	0.4	1.6	3.2	7.8	21.2	25.8	0.2	0.2	0.2
2					1.7	52.5	10.0	35.8	35.5	--	0.2	1.0	4.4	9.0	22.2	20.8	--	0.6	0.6
1	W	M	35	Idiopathic purpura	1.0	57.0	3.0	39.0	23.3	0.2	0.4	0.8	4.8	9.8	19.6	20.0	0.4	1.0	1.0
2					4.7	56.3	5.7	33.3	25.3	--	0.6	0.4	4.4	7.2	16.2	28.6	0.4	0.2	0.2
1	W	M	59	Polycythemia vera	0.7	37.6	1.7	60.0	16.2	--	0.4	0.6	2.6	9.0	7.8	29.0	--	--	--
2					0.7	43.2	2.4	53.7	18.2	--	0.2	0.6	1.8	5.0	5.8	34.0	0.2	--	--
1	C	F	20	Stem cell leukemia	--	46.8	6.6	46.6	91.5	0.2	--	0.2	0.8	5.2	4.0	7.2	--	--	--
2					--	39.0	11.0	50.0	105.4	0.8	--	0.8	1.4	2.8	2.0	5.8	--	--	--
1	C	M	69	Stem cell leukemia	--	45.0	23.0	32.0	147.0	--	--	--	--	0.4	--	4.8	--	--	--
2					--	46.0	22.0	32.0	154.0	0.2	--	--	--	--	0.6	2.4	--	0.2	0.2
1	W	M	6	Stem cell leukemia	--	68.7	2.3	29.0	43.5	0.4	--	--	--	--	--	0.6	--	--	--
2							Insufficient		153.7	0.4	--	--	--	0.4	0.2	0.2	--	0.2	0.2
1	W	M	64	Aleukemic myeloid leukemia	10.0	48.0	10.0	32.0	96.8	--	0.4	0.2	1.8	4.4	11.6	31.8	--	0.8	0.8
2					33.5	28.5	21.5	16.5	308.0	0.4	0.6	1.8	4.4	13.2	23.2	23.4	0.4	0.2	0.2
1	W	M	35	Aleukemic myeloid leukemia	--	69.0	22.0	9.0	218.0	0.6	1.2	4.2	7.8	20.6	28.2	9.8	0.2	0.6	0.6
2					--	75.0	15.0	10.0	209.8	0.2	1.0	5.4	7.6	16.8	22.8	10.8	0.2	0.2	0.2
1	W	M	32	Lympho-sarcoma	1.7	50.0	0.8	47.5	7.5	0.4	--	0.6	2.0	3.0	4.6	19.6	--	0.2	0.2
2					1.3	53.6	0.6	44.5	6.5	0.6	--	0.4	2.2	4.6	11.6	30.2	--	--	--

nique used for obtaining marrow samples was itself a possible source for differences in quantitative values of different cells. They compared the results of marrow samples obtained through biopsies and by punctures. In the latter, the more immature cells and the fixed cells were less numerous than in samples obtained through biopsies. It is possible that the suction force applied at puncture draws back more readily the free cells than the more immature cells, which according to Isaac's conception<sup>13</sup> are probably surrounded by firmer gelatinous substance than the mature cells. These marrow cells were enmeshed by a fine network of the fixed reticulum cells.

Another possible cause for the numerical differences is the unavoidable admixture with red blood cells. The larger the amount withdrawn, the more likely it is that the walls of sinusoids will rupture with resultant admixture with blood. Occasionally, even after withdrawing a small amount of fluid (0.2 c.c.), one may obtain pure blood with apparently no marrow cells present. It is possible that one aspirates the content of a sinusoid.



RACE	SEX	AGE	DIAGNOSIS	PERCENTAGE OF FRACTION OF MARROW				TOTAL NUCLEATED ELEMENTS PER THOUSAND	RETICULO-ENDOTHELIAL CELL	MYELOBLAST	LEUKOBLAST	PRONUCLEOCTE	MYELOCTE	METAMYELOCTE	NEUTROPHILE	EOSINOPHILIC MYELOCTE
				FAT	PLASMA	MARROW	HEMATOCRIT									
C	M	18	Boeck's sarcoidosis	0.5	51.5	6.0	42.0	61.5								
				1.0	49.0	8.5	41.5	92.5	0.2	0.6	1.6	2.2	9.2	15.8	26.6	1.1
W	M	43	Multiple myeloma	1.2	40.4	2.4	56.0	17.0	--	0.2	0.6	3.2	4.4	11.2	31.6	--
				1.0	39.0	2.0	58.0	16.3	--	0.4	1.6	3.1	6.8	14.2	28.4	0.2
W	M	43	Multiple myeloma	0.5	65.0	0.5	34.0	0.94	0.4	0.2	1.0	2.2	5.2	8.0	24.0	--
				Insufficient				1.44	--	--	0.2	1.0	1.4	5.0	43.0	--
W	M	40	Fever (?)	0.7	60.6	2.7	36.0	16.8	--	--	0.6	3.4	6.2	10.2	30.4	0.2
				1.1	50.6	5.0	43.3	56.7	--	0.2	0.8	3.2	8.8	16.0	26.6	0.8
W	M	43	Thrombocytopenia	1.0	63.5	4.5	31.0	55.5	1.8	1.4	1.4	2.8	9.2	10.2	16.4	0.2
				2.2	61.1	7.6	29.1	65.5	2.4	0.2	1.0	2.0	7.0	12.2	16.6	--
W	M	25	Hodgkin's disease	--	60.0	16.0	24.0	103.4	--	0.4	0.8	2.4	7.4	12.4	22.6	0.2
				--	53.8	23.1	23.1	86.1	--	0.2	1.4	4.4	11.4	18.4	13.0	--
W	M	45	Cirrhosis of liver	1.0	56.0	5.0	38.0	49.4	--	0.4	0.8	2.0	12.4	15.0	21.0	0.2
				3.0	52.0	10.0	35.0	82.7	--	0.2	0.8	0.6	8.2	16.8	23.8	--
W	F	54	Bone cyst	3.0	46.0	7.5	43.5	81.6	0.2	0.2	1.2	2.0	6.6	14.0	15.2	--
				2.9	48.8	4.3	44.0	43.8	0.2	0.4	1.0	2.8	6.8	16.6	19.2	--
W	M	30	Lupus vulgaris	6.4	51.4	4.3	31.9	40.2	--	0.2	0.8	2.6	6.6	16.2	24.0	0.6
				6.7	51.7	4.0	37.6	41.5	0.6	0.8	0.8	4.2	10.0	11.8	19.0	1.2

iter,<sup>8</sup> and Jeanneret<sup>9</sup> claimed differences in the cellular composition in different portions of the marrow.

The long bones of man and mammals usually contain yellow fatty marrow which there are scattered centers or islands of hematopoietic cells. However, comparative studies of the bone marrow of flat bones and different portions of the long bones revealed the same trend in the relative percentage distribution of the hematopoietic cells in dogs and monkeys.<sup>2, 3</sup>

Our Cases 8, 14, and 19 illustrate clearly that probably there are irregular islands of fatty tissue in the red marrow also, with marked difference in fat content simultaneously obtained sternal marrow samples. In spite of the numerous differences between the cells in the two simultaneously obtained marrow samples, the trend of the cellular differentiation is the same. This fact is illustrated in Tables I to III by the myeloid-erythroid ratios. If at one portion of the marrow erythroid cells are proliferating, then another portion of the marrow will reveal similar erythroid stimulation. In stem cell leukemias, and in lymphatic leukemias there again was a remarkably similar increase of the abnormal cells counteracted in the different samples. Even in the primary or secondary neoplastic processes of the marrow, with the formation of more or less circumscribed nodular areas, the uninvolved portions of the marrow reveal similar cellular population.

BASOPHILIC MYELOCYTE	BASOPHILIC METAMYELOCYTE	BASOPHILIC	MONOCYTE	LYMPHOCYTE	PLASMA CELL	PRONORMOBLAST	BASOPHILIC NORMOBLAST	POLYCHROMATIC NORMOBLAST	ORTHOCROMATIC NORMOBLAST	NORMOBLAST IN MITOSIS	MYELOID CELL IN MITOSIS	DAMAGED CELL	MEGAKARYOCYTE	IMMATURE LYMPHOCYTE	DAMAGED LYMPHOCYTE	HYDROPHILE	TUMOR CELL	MYELOID-ERYTHROID RATIO
0.2	--	--	1.8	13.4	1.0	0.4	1.0	4.4	11.0	0.2	--	10.6	0.4	--	--	--	--	3.3
--	0.4	--	1.4	12.6	0.4	--	0.6	4.2	12.6	0.2	--	11.0	0.2	--	--	--	--	3.2
0.2	0.2	0.2	1.0	19.2	0.2	0.2	1.2	5.0	5.8	0.2	--	12.2	--	0.6	--	0.2	--	4.4
--	--	--	1.4	17.8	0.8	0.2	1.2	6.0	7.4	0.4	--	8.4	--	0.4	--	0.2	--	3.7
--	--	--	1.0	14.6	0.6	1.0	3.2	6.2	17.2	0.2	--	14.0	--	0.8	--	--	--	1.5
--	--	0.4	1.2	22.2	6.2	0.6	0.8	2.2	5.6	0.4	--	9.6	--	--	--	0.2	--	5.3
--	0.2	--	1.4	25.4	0.4	--	0.6	1.0	9.2	--	--	7.8	0.2	0.4	--	0.4	--	4.9
--	0.2	0.2	0.4	21.2	--	0.2	1.0	1.8	7.0	--	--	8.8	--	1.0	--	--	--	5.9
0.8	0.2	0.6	--	17.2	1.6	1.0	3.4	6.4	11.8	--	--	10.4	0.4	--	--	0.4	--	2.1
0.4	0.2	--	0.2	19.2	2.0	0.2	0.8	4.0	14.8	--	--	13.4	0.8	0.2	--	0.2	--	2.2
--	--	0.2	--	20.2	0.4	--	0.8	1.2	7.6	--	--	12.0	0.4	--	--	0.2	9.6	5.0
--	--	--	0.2	13.4	2.0	--	0.2	1.2	9.4	--	--	11.0	0.6	0.2	--	0.2	10.4	4.8
0.8	0.2	0.2	0.6	9.4	--	0.2	2.4	3.8	11.6	0.8	--	8.4	0.2	0.4	--	2.4	--	3.3
--	0.2	0.2	0.2	11.4	--	0.6	3.6	4.4	16.8	--	--	7.0	--	0.2	--	2.6	--	2.2
--	--	0.2	0.6	21.6	0.6	1.0	0.6	3.8	15.8	0.6	--	12.4	0.6	0.2	--	0.2	--	2.0
--	0.2	--	0.2	19.8	0.2	0.4	0.6	1.8	21.2	0.2	0.2	6.4	--	--	--	--	--	2.0
--	--	0.2	0.4	11.4	0.8	--	0.4	4.8	13.4	--	--	10.6	--	0.2	--	1.0	--	3.1
--	0.6	--	0.6	10.8	0.4	--	0.6	4.6	15.2	0.2	--	11.0	0.2	0.4	--	0.6	--	2.7

Hyperplastic myeloid or erythroid processes, abnormal cell proliferation of acute or chronic leukemic cells will produce uniformly generalized changes in the marrow. Bone marrow punctures with negative results warrant further confirmation and exclusion of technical error.

#### SUMMARY

In a series of different pathologic conditions, two samples of human sternal marrow were aspirated simultaneously. A comparative analysis of the two samples included the percentage distribution of the different fractions of aspirated marrow, the total number of nucleated cells, percentage distribution of the different cells, and the myeloid-erythroid ratio which were computed and tabulated. There were numerous differences between the two samples, but the trend of cellular differentiation was similar as exhibited by the myeloid-erythroid ratios. The morphologic or pathologic appraisal of any one region of the marrow will usually reveal trends in other portions of marrow throughout the body.

#### REFERENCES

1. Stasney, J., and Higgins, G. M.: A Cytologic Study of the Marrow in the Flat Bones of Man, *Folia Haemat.* 61: 334, 1930.
2. Stasney, J., and Higgins, G. M.: A Quantitative Cytologic Study of the Bone Marrow of the Adult Dog, *Am. J. M. Sc.* 193: 462, 1937.

3. Stasney, J., and Higgins, G. M.: The Bone Marrow in the Monkey (*Macacus rhesus*), *Anat. Rec.* 67: 219, 1937.
4. Stasney, J., and Higgins, G. M.: A Quantitative Cytologic Study of the Bone Marrow of the Adult Albino Rat, *Anat. Rec.* 63: 77, 1935.
5. Higgins, G. M., and Stasney, J.: Changes in the Bone Marrow and Peripheral Blood Following Splenectomy in Albino Rats, *Folia haemat.* 56: 189, 1936.
6. Stasney, J., and Higgins, G. M.: The Blood-Forming Organs in Experimental Cirrhosis of the Liver, *Folia haemat.* 55: 93, 1936.
7. Helpap, K.: Zur Kritik der Sternalpunktion, *Klin. Wchnschr.* 16: 558-560, 1937.
8. Reiter, B.: Anatomische Untersuchung zur Frage der Inhomogenität des Knochenmarkes im Hinblick auf die Auswertung der Sternalpunktion, *Ztschr. f. d. ges. exper. Med.* 103: 694-703, 1938.
9. Jeanneret, H.: La moelle osseuse en clinique et à la salle d'autopsie; étude comparative, *Schweiz. med. Wchnschr.* 70: 351-357, 1940.
10. Reich, C., and Kolb., E. M.: A Quantitative Study of the Variations in Multiple Sternal Marrow Samples Taken Simultaneously, *Am. J. M. Sc.* 204: 496, 1942.
11. Limarzi, L. R.: The Diagnostic Value of Sternal Marrow Aspirations, *Illinois M. J.* 75: 38, 1939.
12. Dameshek, W., Henstell, H. H., and Valentine, E. H.: The Comparative Value and the Limitations of the Trephine and Puncture Methods for Biopsy of the Sternal Bone Marrow, *Ann. Int. Med.* 11: 801, 1937.
13. Isaacs, R.: The Physiologic Histology of Bone Marrow; the Mechanism of the Development of Blood Cells and Their Liberation Into the Peripheral Circulation, *Folia haemat.* 40: 395, 1930.
14. Custer, R. P.: Studies on the Structure and Function of Bone Marrow; Variability of the Hemopoietic Pattern and Consideration of Method for Examination, *J. LAB. & CLIN. MED.* 17: 951, 1932.

# THE RETICULO-ENDOTHELIAL ORIGIN OF THE BONE MARROW PLASMA CELLS IN HYPERSENSITIVE STATES

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## INTRODUCTION

SINCE its description by Cajal in 1890, the plasma cell has been observed by pathologists in the hematopoietic tissue and in the specific lesions of varying clinical conditions. Huebschmann (1913) studied the spleens of patients dying of various infectious diseases and observed a splenic plasmacytosis resulting from pneumococcic, staphylococcic, diphtheric, and typhus infections. He found little or no increase in splenic plasma cells in cases of fatal miliary tuberculosis, anthrax, or in a luetic stillborn—all overwhelming infections. He associated the plasma cell with the production of antibodies.

Perlzweig, Delrue, and Geschickter (1928) and others since then have associated multiple myeloma, which is apparently a malignancy of the bone marrow resulting in the accumulation of plasma cells, with hyperglobulinemia. Since that time a number of investigations have related the plasma cell to hyperglobulinemia in clinical disease: Osgood and Hunter (1934) in plasma cell leukemia, Markoff (1937) in serum sickness, and Bing and Plum in agranulocytosis (1937). Bing, in various acute and chronic infections, myelomatoses, and other diseases (1940), concluded that the plasma cell of reticulo-endothelial origin was the source of the blood globulin. Fleischhacker (1940 and 1941) made many observations on patients with hyperglobulinemia and found that the bone marrow revealed simultaneous plasmocytosis. His conclusions were similar to those of Bing. Gormsen and Heintzelmann (1941) reported studies of blood globulin and sternal marrow in patients with serum sickness; they found a distinct parallelism between increased marrow plasma cells and hyperglobulinemia and stated that their observations support Bing's theories. Taussig and Somogyi (1940) reported hyperglobulinemia in six cases of granuloma inguinale and stated that it is common in lymphogranuloma inguinale. The presence of plasma cells in the lesions of these two diseases is well known. Kagan (1943) has furnished additional clinical evidence of the relation of globulin level and plasmacytosis.

The relationship of plasmacytosis to globulin and antibody production is substantiated by the experimental literature. Downey's (1911) description of a plasmacytosis in the bone marrow of a rabbit with an incidental nonspecific subcutaneous abscess initiates the series. Doan, Sabin, and Forkner (1930) and Miller (1931) produced omental plasmacytosis by repeated intraperitoneal administration of tuberculo proteins. Rich (1935 and 1936) produced acute splenic tumor in rabbits by horse serum. The characteristic feature was the "acute splenic tumor cell." In similar preparations made in this laboratory we have identified these cells as developing plasma cells. Kolouch (1938)

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studied the plasma cell and found it in the bone marrow of ten species of laboratory animals and also showed that the plasma cell content of the rabbit bone marrow could be experimentally increased by sensitization and shock with *Streptococcus viridans*. He demonstrated the development of the bone marrow plasma cell from the reticular plasma cell in a period of five days and postulated a relation between this transformation and the development of antibodies. Björneboe and Gormsen (1941) described the experimental production of a hyperglobulinemia in rabbits by intensive immunization with different types of formalin-killed pneumococci. They found a concomitant increase in plasma cells in various tissues. They postulated that the increase in the globulin fraction of the blood was due to the increase in antibodies and that this in turn was related to plasmacytosis.

The purpose of the following experiments is to elucidate the relationship of the bone marrow plasma cells to the reticulo-endothelial cell and to the phenomena of sensitivity and shock. Both bacterial and simple protein antigens have been used. Illustrative material from a case of serum sickness is included.

#### MATERIALS AND METHODS

Rabbits were used in all experiments. Series 1\* consists of six rabbits which were injected repeatedly over a period of several months with varying doses of *Str. viridans*. Series 2 contains three rabbits injected subcutaneously with a single dose of 10 c.c. of a culture of *Str. viridans* suspended in agar. After a period of five weeks they were shocked with broth culture of the same organism and serial rib biopsies were made at intervals up to five days. Series 3 consists of sixteen animals which were sensitized to egg white by its injection on alternate days in the following dosages: 1 c.c., intravenously; 0.5 c.c., intravenously; and 1 c.c., intramuscularly. Four animals were killed following the sensitization and four immediately after shock, which was induced twenty to thirty days later by injection of 0.4 c.c. egg white intravenously; four were killed on the fourth day following shock by injection 0.4 c.c. egg white; while the bone marrow of the remaining four was sampled before sensitization, after sensitization, following anaphylactic shock, and periodically thereafter for four days.

The bone marrow was imprinted and stained with Wright-Giemsa.

#### EXPERIMENTAL RESULTS

The six rabbits of series 1 which were treated over a period of months with multiple injections of *Str. viridans* all showed marked increase in the bone marrow plasma cells, and in some animals there was noted an increase in the reticular plasma cells. These changes were critically followed in the three animals of the second series. Rib bone marrow biopsies were made before sensitization and the elements compared with similar material from the same subjects after sensitization and following shock. The control bone marrows show few reticular plasma cells. These are found to be increased in the biopsies taken after sensitization. They appear as large cells with basophilic, rather muddy cytoplasm with eccentric nuclei characterized by a nuclear pattern similar to that seen in reticular cells (Fig. 2,a).

In these preparations the chromatin appears as a fine purplish-violet dust sprinkled on a background of pinkish-yellow karyoplasm (parachromatin). The chromatin granules are uniform, finely divided, round purplish-violet particles.

\*The material for this series was obtained from Dr. B. J. Clawson.

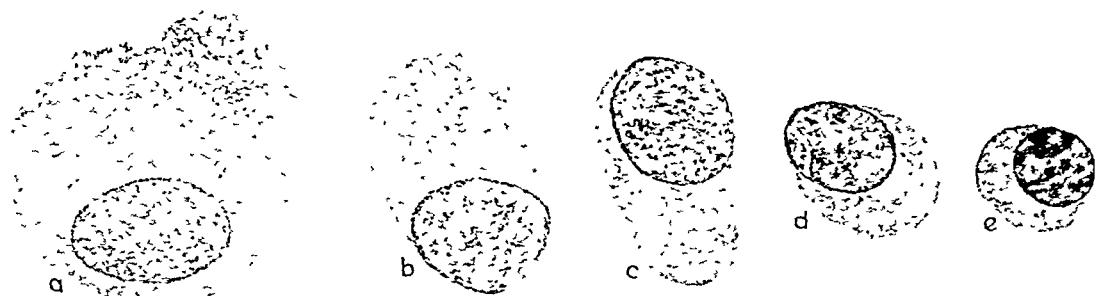


Fig. 1.—Representative plasma cells imprinted at biopsy from bone marrow of rabbit sensitized to egg white. *a*, Reticular plasma cell before anaphylactic shock. *b*, Initial transformation stage four hours after shock. *c*, Additional shrinkage and metamorphosis after eight hours. *d*, Medium-sized plasma cell at forty-eight hours. *e*, Typical Marshalko type plasma cell after ninety-six hours.

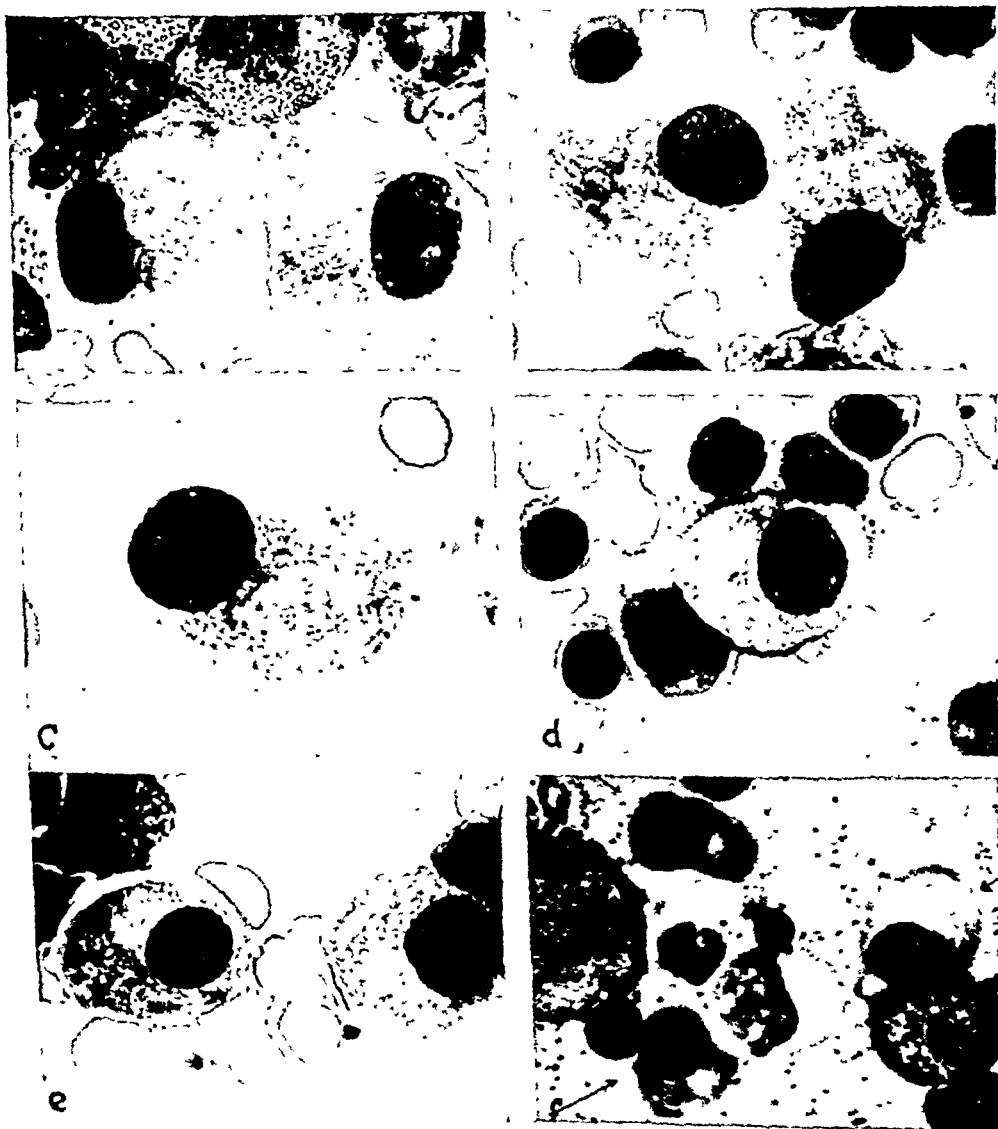


Fig. 2.—Photomicrographs of cells showing stages in metamorphosis from reticular plasma cells to Marshalko type plasma cells. Rabbits sensitized and shocked with *Str. viridans*. *a* and *b*, Group of plasmacellular reticulum cells before shock. *c* to *f*, Transformation stages to Marshalko type plasma cells.

Each one is surrounded by and sharply demarcated from the parachromatin which it tends to mask. The chromatin pattern is more condensed and the nuclear membrane slightly thicker than in the unaltered reticulum cell. In the cytoplasm the basophilic spongioplasm occurs as minute masses which are arranged to form small coalescing bars. These elongated clumps are surrounded by and sharply separated from an abundant yellowish hyaloplasm. The arrangement of these two components of the cytoplasm gives it its characteristic canaliculated appearance. In the region of the nucleus there is frequently seen a lighter staining area (increase of hyaloplasm) which suggests a Hof. When occurring alone the cells appear irregularly elliptical, but most frequently they are seen in clumps of five to fifteen cells, the cytoplasmic boundaries then being rather indistinct.

The initial transitional stage occurs within two hours after shock (Fig. 2, *b*). The boundary of the cytoplasm becomes more clearly defined and rounding of the cells is initiated. In the nucleus contraction occurs and a coarser chromatin pattern is to be observed. At four hours shrinkage of the cytoplasm is noted and the basophilia is more apparent than before. By this time dispersion of the cells which previously had been clumped has taken place, and the reticular plasma cells are mixed with the other bone marrow elements. Shortly after four hours the Hof becomes marked. This together with the more condensed nucleus and increased basophilia of the spongioplasm leads to cells more easily recognizable as developing plasma cells. These cells become abundant at five hours. In the ensuing five hours, many of these transform to large plasma cells (Fig. 2, *c*). These are characterized by the eccentric nucleus, a heavier nuclear membrane, blocklike arrangement of the chromatin masses, and a distinct Hof in a still more basophilic cytoplasm. These large plasma cells measure about 20  $\mu$  in diameter. Their further evolution consists of shrinkage in the next twelve to thirty-six hours to medium-sized plasma cells measuring about 12  $\mu$  in diameter (Fig. 2, *d* and *e*). With this decrease in size, there is accentuation of the nuclear pyknosis, the cytoplasmic basophilia, the Hof, and the initial appearance of the radkern nuclear pattern. These cells are abundant two days following the shock. Continuation of these tendencies results in the small plasma cell (the true Marschalko type) abundant at four days (Fig. 2, *f*). Some of these cells become, after the fifth day, extremely pyknotic and apparently degenerate, their nuclear structure being obscured by their staining properties.

At no time during this cycle does the reticular plasma cell exhibit phagocytic properties, though other cells of the reticulum are seen to have ingested bacteria and erythrocytes.

To serve as control for the bacteremia and local infection of the first two series, as well as to test the general nature of the phenomenon, the sixteen rabbits of series 3 were sensitized to egg white and studied before and after shock. The observations differ little from those obtained using *Str. viridans* as antigen. In the four animals killed before shock, a massive increase of plasma-cellular reticulum cells may be seen (Fig. 3, *a*). As before, the cells appear in clumps and show morphology identical to those previously described. In the four animals that died immediately upon shock, no change from the pre-

shock picture is noted. Those which were killed four days after shock show marked plasmacytosis of the bone marrow (Fig. 3, *b*). Serial biopsies of the remaining four at 2,4,8,14,24,48,72, and 96 hours, as well as pre- and post-sensitization controls, confirm in all respects the transformation from the reticular plasma cell to the plasma cell previously described (Fig. 1).

That this same transformation from reticular plasma cell to plasma cell occurs in human beings is shown in Fig. 4, which is a blood smear taken from a boy suffering from serum sickness resulting from the administration of tetanus antitoxin.

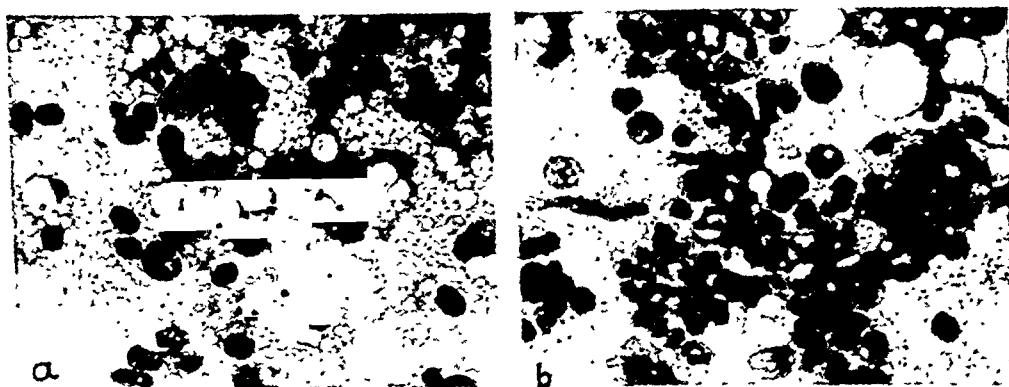


Fig. 3.—Bone marrow imprints from rabbit sensitized to egg white. *a*, Clumped reticular plasma cells before shock. *b*, Marshalko type plasma cells four days after shock.

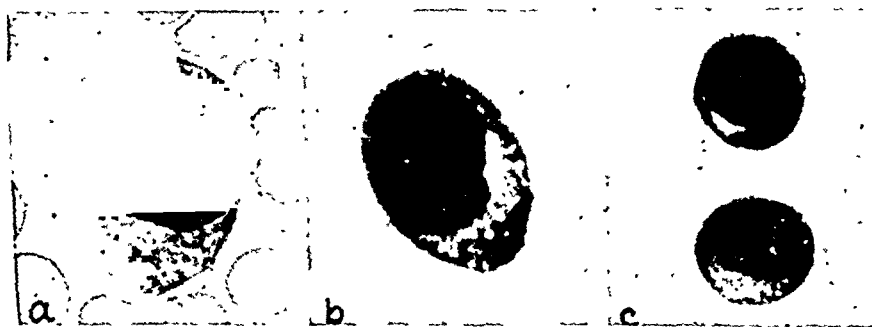


Fig. 4.—Illustration of intermediate stages between reticular cells and plasma cells as seen in blood smear of patient with serum sickness. *a*, Cell with reticular type nucleus. *b*, Condensation of nucleus. *c*, Further condensation of nucleus and cytoplasm to close approximation of plasma cell.

#### DISCUSSION

These experiments show two phenomena of interest. There is an increase in a specific type of bone marrow reticulum cell following sensitization to an antigen. Second, anaphylactic shock initiates a morphologic cycle by which the reticular plasma cells evolve to plasma cells. We have noted the development of similarly striking plasmacytosis in the other reticulo-endothelial organs following sensitization and shock with bacterial and other antigens. In view of the evidence in the literature and our own experiments, it is highly suggestive that the transformation of the plasmacytic reticulum cell to the Marshalko type

plasma cell is intimately associated with the production of antibody protein. It is consistent with recent findings (Dougherty and White, 1946, Dempsey and Wislocki, 1946) to presume that the basophilia of the early plasmaocyte may be associated with heightened protein formation. In view of the needs of the organism at this time, antibody production might well be carried on in such active cells. Certain it is that the loss of volume is a most striking cellular change in the procession of stages from basophilic reticulum to small plasma cell. Yet, the impression is gained that stainable material in these preparations is not reduced. A loss of the fluid components of the cell is obvious. It would be hard to overlook, however, the possible relation of the antigen-antibody reaction to the permeability of the cell membrane in searching for a possible mechanism for these changes. If serum hyperglobulinemia is directly related to plasma cell development, it is likely that some of the cell shrinkage may be due directly to loss of protein elements in the form of globulin. Morphology reaches its limitations in this type of work and must be supplemented by other methods. Before a thorough understanding of this matter is to be had, we must know whether, in tissue culture, the cell changes under question are associated with the production of antibodies. In addition, the role of the various cell organelles in the reaction to anaphylactic shock must be worked out on a cytochemical basis.

Another aspect of the problem which must remain speculative until more detailed information is available is the nature of the stimulus furnished by anaphylactic shock, which brings about the sequence of cell transformations that produces Marshalko plasma cells from a much less differentiated type of cell. The spectacular operation of this cytomorphosis not only serves to illustrate the exquisite relation between physiologic changes and the ontogeny of cellular elements, but it also promises to serve as an experimental preparation for the determination of the relation of nuclear changes to cytoplasmic activity.

Markoff's work on serum sickness, as well as our case, serves to illustrate the clinical importance of the matter under discussion. It is apparently the exceptional case in which plasma cells are found in the peripheral blood, but the concomitant appearance of circulating reticular plasma cells indicates that the process in the bone marrow is essentially the same as in the bone marrows of experimental animals. It should be noted that in the rabbits, circulating plasma cells were not regularly found, and we may believe that some secondary factor in our clinical case brought the cells into the peripheral blood.

#### SUMMARY

1. An analysis of the literature provides ample evidence to link plasmacytosis to antibody production.

2. With *Str. viridans* and egg white as antigens, sensitization of rabbits was found to increase enormously the number of reticular plasma cells in the bone marrow.

3. Shocking the animals with the antigen initiated a cycle of nuclear and cytoplasmic condensation, visible in two hours and extending to four days, by which the reticular plasma cells are transformed to Marshalko type plasma cells,

4. In a case of human serum sickness, both reticular plasma cells and various stages of the plasma cell appeared in the peripheral blood.
5. It is suggested that this morphologic cycle is associated intimately with the production of antibodies.

## REFERENCES

- Bing, J.: Further Investigations on Hyperglobulinemia (Is Serum-Globulin Formed From Plasma Cells and Reticulo-Endothelial Cells?), *Acta med. Scandinav.* 103: 565-583, 1940.
- Bing, J.: Further Investigations on Hyperglobulinemia (Occurrence and Degree of Hyperglobulinemia in Various Diseases. Ratio Between Hyperglobulinemia, Hyperproteinemia, and Hypoalbuminemia. Formolgel-Reaction), *Acta med. Scandinav.* 103: 547-564, 1940.
- Bing, J., and Plum, P.: Serum Proteins in Leucopenia. (Contribution on Question About Place of Formation of Serum Proteins), *Acta med. Scandinav.* 92: 415-428, 1937; also, *Ugesk. f. læger* 99: 738-743, 1937.
- Bjørneboe, M., and Gormsen, H.: Preliminary Report on Investigations on Occurrence of Plasma Cells in Experimental Hyperglobulinemia in Rabbits, *Nord. med. (Hospitalstid.)* 9: 891-894, 1941.
- Bjørneboe, M., and Gormsen, H.: Untersuchungen über das Vorkommen von Plasmazellen bei experimenteller Hyperglobulinämie bei Kaninchen. (Vorläufige Mitteilung), *Klin. Wchnschr.* 20: 314-316, 1941.
- Bjørneboe, M., and Gormsen, H.: Experimental Studies on the Role of Plasma Cells as Antibody Producers, *Acta Path. et microbiol. Scandinav.* 20: 649-692, 1943.
- Cajal, S. R.: *Manual de anatomia patologica general*, ed. 1, Barcelona, 1890.
- Dempsey, E. W., and Wislocki, G. B.: Histochemical Contributions to Physiology, *Physiol. Rev.* 26: 1-27, 1946.
- Doan, C. A., Sabin, F. R., and Forkner, C. E.: Reaction of the Connective Tissues of the Normal Rabbit to Water Soluble Protein and Polysaccharide, *J. Exper. Med.* 52: (suppl. 3) 73-86, 1930.
- Doughterty, T. F., and White, A.: Increased "Plasma Cell" Production Following Adrenal Cortical Stimulation, *Anat. Rec.* 94: 13-14 (Suppl.), 1946.
- Downey, H.: The Origin and Structure of the Plasma Cells of Normal Vertebrates, Especially of the Cold Blooded Vertebrates and the Eosinophils of the Lung of Amblystoma, *Folia haemat.* 11: 275-314, 1911.
- Fleischhacker, H.: Ueber die Bedeutung der Reticuloendothelien und Plasmazellen des Knochenmarkes, *Ergebn. d. inn. Med. u. Kinderh.* 60: 508-564, 1941.
- Fleischhacker, H.: Ueber die Plasmazellen und das reticuloendotheliale System des Knochenmarkes. Beitrag zur Herkunft der Plasmaeiiweisskörper, *Deutsches Arch. f. klin. Med.* 186: 506-523, 1940.
- Gormsen, H., and Heintzelmann, F.: Behavior of Sedimentation Reaction, Serum Proteins, and Sternal Punctate in Serum Sickness, *Nord. med. (Hospitalstid.)* 11: 2125-2128, 1941.
- Huebschmann: Das Verhalten der Plasmazellen in der Milz bei infektiösen Prozessen, *Verhandl. d. deutsch. Path. Gesellsch.* 16 Tag, Marburg, pp. 110-114, 1913.
- Kagan, B. M.: Hyperglobulinemia, *Am. J. M. Sc.* 206: 309-315, 1943.
- Kagan, B. M.: Studies on Clinical Significance of Serum Proteins, *J. LAB. & CLIN. MED.* 27: 1457-1463, 1942; correction 28: 254, 1942.
- Kolouch, F.: Origin of Bone Marrow Plasma Cell Associated With Allergic and Immune States in Rabbits, *Proc. Soc. Exper. Biol. & Med.* 39: 147-148, 1938.
- Kolouch, F.: A Study of the Bone Marrow Plasma Cell of Mammals With Special Reference to Its Origin in the Rabbit Under Normal and Experimental Conditions, Thesis, University of Minnesota, 1938, pp. 1-84.
- Markoff, N. G.: Die Reticuloendothelien des Knochenmarks, beurteilt durch Sternalpunktion, *Deutsches Arch. f. klin. Med.* 180: 530-538, 1937.
- Miller, F. R.: Induced Development and Histogenesis of Plasma Cells, *J. Exper. Med.* 54: 333-348, 1931.
- Osgood, E. L., and Hunter, W. C.: Plasma Cell Leukemia, *Folia haemat.* 52: 369-383, 1934.
- Perlzweig, W. A., Delrue, G., and Geschickter, C.: Hyperproteinemia Associated With Multiple Myelomas; Report of Unusual Case, *J. A. M. A.* 90: 755-757, 1928.
- Rich, A. R.: Inflammation in Resistance to Infection, *Arch. Path.* 22: 228-254, 1936.
- Rich, A. R.: Acute Splenic Tumor Produced by Non-bacterial Antigens, *Proc. Soc. Exper. Biol. & Med.* 32: 1349-1351, 1935.
- Taussig, A. E., and Somogyi, M.: Hyperglobulinemia in Granuloma Inguinale, *J. LAB. & CLIN. MED.* 25: 1070-1071, 1940.

# HEMATOPOIESIS IN THE BONE MARROW OF RATS RECOVERING FROM NUTRITIONAL ANEMIA

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## INTRODUCTION

THE present paper is a report on the results of studies of the hematopoietic activities found in the bone marrow of rats made anemic by being fed a diet composed of milk only and then caused to recover from the anemia by being fed copper and iron as supplements to the milk diet. Studies of the recovery of rats made anemic by this method have seldom included observations on changes in the number of leucocytes but have chiefly been concerned with the regeneration of hemoglobin and erythrocytes and with the agents which induce regeneration of those blood elements. The level of hemoglobin and the number of erythrocytes of the circulating blood have been employed as indicators of the severity of the anemia produced by feeding the milk diet, and the rate and degree of regeneration of those hematologic elements have been used to measure the curative properties of substances added to the milk diet. Through the work of Hart, Steenbock, and Elvehjem and co-workers,<sup>1-5</sup> Beard and Myers and associates,<sup>10-17</sup> Mitchell and Miller,<sup>18</sup> Hamre and Miller,<sup>19, 20</sup> Smith and Otis,<sup>21</sup> Stein and Lewis and co-workers,<sup>22-25</sup> and others, it has been established that copper and iron when fed simultaneously in adequate quantities as supplements to the milk diet will cause recovery from the anemia.

Studies of the blood-forming organs of rats in nutritional anemia and of rats recovering from the anemia have been few in number. Von Haam and Beard<sup>26</sup> found the spleen of rats made anemic by being fed a diet of milk only to be small, pale, and soft. The spleens of the anemic animals possessed fewer and smaller follicles, a decreased perifollicular zone, and pulpar cells which showed signs of degeneration. Hamre and Miller<sup>19</sup> also found the spleens of anemic rats to be small in size and to possess a decreased amount of white pulp tissue, the decrease in the extent of the tissue being due to a loss of the outer zone and part of the inner zone of the Malpighian corpuscles and of the outer portions of the periarterial white pulp. The red pulp was increased in area and included circulating mature red blood cells and normal reticular cells. There was no indication of cell multiplication in the spleens of the anemic animals. The latter authors also demonstrated that, during the recovery from the anemia following iron and copper therapy, the spleens of the animals became temporarily enlarged and mottled with grayish-white hematopoietic areas. When approximately normal values of the blood were restored, the spleens of the recovering animals decreased to normal size. The white pulp

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tissues were regenerated and restored to normal histologic appearance during the period of enlargement, and the normal appearance of the white pulp was retained when the spleens returned to normal size. Active erythropoietic tissue occupied the red pulp areas of the enlarged spleens, and in the early periods of recovery those areas included large numbers of hemocytoblasts while in the later periods of recovery the areas were composed largely of polychromatophilic normoblasts. The erythropoietic areas decreased in extent and disappeared in the later periods of recovery. In a later study Hamre and Miller<sup>20</sup> demonstrated that removal of the spleens of anemic animals did not prevent the recovery of hemoglobin and erythrocytes under copper and iron therapy. However, there were indications that the rate of recovery of the erythrocytes was slower for the splenectomized than for the nonsplenectomized animals.

The paper by Fitz-Hugh and associates,<sup>27</sup> as far as the present writer has been able to determine, is the only paper in the literature on the nutritional anemia of the rat which presents observations on some of the characteristics of the bone marrow of anemic animals and of animals recovering from the anemia. These authors made observations on the quantity of fatty and cellular tissue of the marrow and pointed out that the cellularity of the marrow of normal animals was greater than that of anemic animals. They also noted that the addition of curative agents to the milk diet caused an increase in the cellularity of the marrow of all of their treated animals. For animals fed iron alone as supplement to the milk diet, the hyperplasia of the marrow tissue persisted throughout a seventy-day period of therapy. The marrow of anemic animals fed iron and copper, or iron and sodium glutamate, became highly hyperplastic in the early stages of recovery but in the later stages became less cellular than the marrow of normal animals. Though the paper<sup>27</sup> did indicate that gross microscopic changes of the bone marrow of anemic animals did follow therapy, it did not describe the cytology of the marrow tissues or point out the nature of the hematopoietic response to therapy.

Because of the marked hematopoietic response of the spleen and the rapid restoration of normal blood values which followed the feeding of therapeutic agents, the present writer assumed that the bone marrow of anemic animals similarly would exhibit a sharp hematopoietic response to therapy. Preliminary studies of the bone marrow of animals killed at daily intervals during recovery from anemia were carried out, and, though an increase in the cellularity of the marrow was found, the cytologic characters noted indicated that only hematopoiesis of the homoplastic type occurred. However, the observations of Hamre and Miller<sup>20</sup> that marked increases of normoblasts and leucocytes of the circulating blood occurred on the second and the fifth or sixth day of therapy suggested that a marked change of hematopoiesis of the bone marrow might occur during the first day of feeding of the iron and copper supplements. Study of the bone marrow of rats killed at hourly intervals during the first two days of supplement feeding did show a marked hematopoietic response to the therapy. The present paper describes the hematologic changes found to occur in the bone marrow and the blood of anemic animals during the first forty-eight hours of the recovery period.



## MATERIALS AND METHODS

Sixty animals divided into three groups of twenty each were observed in these studies. Though the three groups of animals were placed on experiment at different times, to establish identical conditions for study, all animals were managed in the same manner. All animals were made anemic by the method outlined by Elvehjem and Kemmerer,<sup>28</sup> that is, at the age of 21 days they were weaned and placed on a diet of cow's milk. The milk was obtained at the University Farm and was collected directly into glass bottles and fed to the animals in glass dishes. The animals were kept in individual galvanized iron wire cages. When the hemoglobin value had decreased to 3.5 Gm. or less, per 100 c.c. blood, the animals were given iron and copper as supplements to the milk. Eighteen of the animals of the first group were fed the iron and copper supplements after 28 to 32 days on the milk diet while the remaining two animals of the group were kept on the milk diet for 44 days. The animals of the second and third groups, though they possessed satisfactorily low hemoglobin values after 28 days on the milk diet, were continued on the deficient diet for 36 to 54 days in the hope that the hemoglobin and erythrocyte values would be further reduced and a more severe anemia established. Though the animals continued on the milk diet for the longer periods did become progressively weaker, only slight decrease of hemoglobin and erythrocyte values occurred for some but not all animals. Each animal fed the copper and iron supplements received a single dose of the curative agents unless they were continued under observation for more than 24 hours when they were given a second dose of the supplements at the beginning of the second 24-hour period. A single dose of the curative agents consisted of 0.5 mg. iron for all individuals and copper fed at the rate of 0.003 mg. per gram body weight. The iron was supplied as ferric chloride ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) and the copper as cupric sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ). The copper and iron content of the various solutions used were checked by the colorimetric method.

The first group of twenty animals was employed as a part of a preliminary experiment to determine approximately at what time during the early stages of recovery critical or significant phases of hematopoiesis might occur. Because of the erythropoietic function of the spleen during recovery, it was decided that anemic animals with large, actively erythropoietic spleens should not be used in this study. Each animal, therefore, was subjected to laparotomy and the size and appearance of the spleen were noted. All animals were used in the experiment because the spleens were small or medium sized and none possessed grayish-white erythropoietic areas. Two of the animals, to serve as controls, were killed without having received copper and iron supplements. Seventeen of the animals were fed iron and copper and one of these was killed at each of the following hours after copper and iron feeding: 2, 3, 5, 7, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 36, and 48 hours. The twentieth animal of this group was bled hourly from the fourteenth to the twenty-eighth hour after feeding to provide hourly blood values.

Since none of the animals of the first group possessed large and actively erythropoietic spleens and laparotomy introduced a complicating factor into the experiments, none of the animals of the second and third groups were subjected to exploratory operation. Five of the animals of these groups were killed as anemic control animals. Since the animal of the first group killed at 22 hours after iron and copper feeding possessed a bone marrow showing a particularly sharp response to therapy, the animals of the second and third groups and which received iron and copper, were killed at some hour between 10 and 48 hours after supplement feeding.

Hemoglobin, erythrocyte, leucocyte, and platelet values were determined for all control animals and for all treated animals at the time they were given their first dose of copper and iron and again at the time they were killed. All blood samples were obtained from the rat's tail by the usual methods. Hemoglobin values were determined by the acid hematin method employing a Klett-Summerson photoelectric colorimeter. The erythrocyte and leucocyte counts were made by the usual methods and the platelet counts by the method of Reese and Ecker. At the time blood samples were taken for blood values, blood smears were also prepared. The blood smears were stained with Wright's stain and Giemsa stain. At the time the animals were killed, the femur, humerus, vertebrae of the base of the tail,

and vertebrae from the middle of the free portion of the tail were cleaned of muscles and connective tissue and fixed in Helly's Zenker-formol fixative. The marrow tissue of one femur was removed and a portion used to prepare smears and imprints and the remainder fixed for sectioning. The smears of the bone marrow were stained with Wright's stain followed by Giemsa stain. Entire bones and the marrow removed from the femur fixed for histologic sections were embedded in celloidin, decalcified, sectioned, and stained in hematoxylin eosin, hematoxylin-eosin-azure II, or Dominici's toluidine blue-eosin-orange G stain.

#### OBSERVATIONS AND RESULTS

The results of this investigation will be presented in two parts, the first dealing with the values for the hemoglobin and blood cells of anemic animals and animals given iron and copper supplements and the second with the histology of the bone marrow of anemic and treated animals. Observations on the values of the blood elements are presented for the purpose of indicating the characteristics of the anemia investigated in this study and because the changes of the values of the blood elements which follow treatment with copper and iron help explain the histologic changes found in the bone marrow. The relation of the hematologic conditions observed to the controversial problems and theories of hematopoiesis will be presented in the discussion which follows this section of the paper.

The anemic animals were physically weak and inactive and many of the animals, often in spite of high platelet counts, showed a tendency toward failure of blood coagulation, the bleeding from the tail after taking blood samples being difficult to control. The average values and range of values of the blood elements of the anemic animals are summarized in Table I. The range of values recorded for hemoglobin and erythrocytes and the presence of normoblasts in the blood smears indicate that the anemia for all animals was a severe one. Though the total number of leucocytes was reduced below normal, the absolute values given for the various types of leucocytes show that the differential leucocyte count was approximately normal and the lymphocyte the predominant leucocyte type of the blood smear. The platelets, as shown by the range of values listed in Table I, were extremely variable in number for our anemic animals.

The anemia produced by feeding milk to the rats of this experiment was of the microcytic, hypochromic type. Anisocytosis was marked and the blood smears showed quite clearly that the greater number of erythrocytes was smaller than normal. An average of  $4.5 \mu$  was obtained by employing a diffraction erythrocytometer for measuring the diameter of the erythrocytes of the blood smears of thirty-six of the anemic animals. Though there is no agreement in the published values for the diameter of the red cells of normal rats, the values of  $5.7 \mu$  given by Kleiberger,<sup>29</sup>  $6.3 \mu$  given by Scarborough,<sup>30</sup> and  $6.8 \mu$  given by Gulliver and quoted by Ponder<sup>31</sup> are all greater than the average diameter obtained for our animals. The erythrocytes of the blood smears were either strongly basophilic, polychromatophilic, or possessed a faintly stained narrow acidophilic margin. Basophilic stippling of the red cells was present and Jolly bodies were encountered quite frequently in the blood smears. Poikilocytosis was either absent or slight.

TABLE I. SUMMARY OF BLOOD VALUES OF ANEMIC RATS AND RATS AFTER COPPER AND IRON THERAPY

BLOOD ELEMENT	AVERAGE VALUES*		RANGE OF VALUES*	
	ANEMIC ANIMALS ON DAY KILLED AS CONTROLS OR FED COPPER AND IRON SUPPLEMENT	TREATED ANIMALS KILLED FROM 2 TO 48 HOURS AFTER FIRST FEEDING OF COPPER AND IRON	ANEMIC ANIMALS ON DAY KILLED AS CONTROLS OR FED COPPER AND IRON SUPPLEMENT	TREATED ANIMALS KILLED FROM 2 TO 48 HOURS AFTER FIRST FEEDING OF COPPER AND IRON
Hemoglobin in grams per 100 c.c. blood	2.8 (54)†	3.3 (46)‡	Less than 2.0 to 3.8 (59)	Less than 2.0 to 5.2 (50)
Erythrocytes in millions per c.mm. blood	2.05 (59)	2.22 (52)	1.16 to 2.92 (59)	1.29 to 3.41 (52)
Leucocytes in thousands per c.mm. blood	5.37 (57)	16.44 (50)	2.71 to 11.65 (57)	3.16 to 42.71 (50)
Platelets in thousands per c.mm. blood	409 (40)	433 (36)	96 to 814 (40)	80 to 926 (36)
Normoblasts in number per c.mm. blood	208 (57)	2121 (50)	0 to 2063 (57)	0 to 8426 (50)
Heterophile in number per c.mm. blood	2060 (57)	10,171 (50)	441 to 4943 (57)	1408 to 32,708 (50)
Eosinophiles in number per c.mm. blood	78 (57)	429 (50)	0 to 331 (57)	0 to 2431 (50)
Lymphocytes in number per c.mm. blood	3433 (57)	5359 (50)	1005 to 6156 (57)	1256 to 17,738 (50)

\*Numbers in parentheses indicate the number of animals observed for each blood element.

†Five additional animals had less than 2.0 Gm. hemoglobin per 100 c.c. blood and, therefore, below the lowest calibration of the colorimeter. These animals were not included in the computation of the average value.

‡Four additional animals had less than 2.0 Gm. hemoglobin per 100 c.c. blood and, therefore, below the lowest calibration of the colorimeter. These animals were not included in the computation of the average value.

In this study the anemic animals given recovery treatment were killed a few hours after the initial copper and iron feeding and, therefore, did not show marked improvement in general physical condition. However, the animals continued to the end of the 48-hour observation period were more active than before receiving copper and iron. The anisocytosis did not disappear and in some instances was more marked after therapy than before. Likewise, the microcytosis was continued, the average diameter of the erythrocytes of thirty-six treated animals being  $4.5 \mu$ . The basophilic erythrocytes in some instances were increased in number and in general the hypochromasia continued unchanged after therapy. From these observations it is apparent that for the period of the first 48 hours after the initial copper and iron feeding, the hypochromic and microcytic character of the anemia was continued and was not markedly altered.

The values for the hemoglobin and blood cells of the animals fed copper and iron are summarized in Table I. Though the average values and range of values presented in the table are for all animals fed the curative substances and does not take into consideration the fact that the animals were killed at various times after supplement feeding, they do indicate the character of the response to therapy. Slight increases in value are indicated for hemoglobin, erythrocytes, and platelets, while marked increases in number are shown for normoblasts and leucocytes. The great increase in number of white blood cells is shown to be due primarily to an increase in number of heterophile leucocytes.

Though lymphocytes were increased over their values for untreated anemic animals, their increase in number was small when compared to the increase in number of heterophile leucocytes. As a result the differential leucocyte count was altered, the heterophile becoming the predominant leucocyte type after treatment. The marked increase in number of normoblasts, eosinophiles, and heterophiles shows that the first response to copper and iron feeding is myeloid in nature and characterized by a discharge of those cells into the circulating blood by the bone marrow.

Examination of the hemoglobin and blood cell values for animals killed at different hours after the initial feeding of copper and iron shows that a latent period of no regular gain immediately follows the feeding. It also shows that the latent period continues to 26 hours for hemoglobin and 28 hours for erythrocytes, while for normoblasts and leucocytes it continues only to 12 hours after feeding. The duration of the latent period is shown particularly well by the gain or loss in values shown in the graphs of Figs. 1 and 2. Since the platelets did not show a definite regular response to therapy, their changes in values were not graphed and included in the illustrations.

Though Krumbhaar,<sup>32</sup> Wintrobe,<sup>33</sup> and others state that due to an increase in number of neutrophiles, a postoperative leucocytosis is found to occur, the values for leucocytes of our animals subjected to laparotomy do not differ significantly from the values of animals not subjected to operation. We have concluded that the change in numbers of normoblasts and leucocytes of the circulating blood of our animals was due to the copper and iron feeding. We have also concluded that the increase in number of those cells was due to their discharge by the bone marrow and that they were discharged during the period of high and low values extending through the period between 12 to 26 hours after copper and iron feeding. That period we regard to be a critical period of response to therapy and, as will be described, is accompanied by critical changes of hematopoiesis in the bone marrow.

The bones whose marrow tissues were observed for histologic changes in this investigation, on the basis of position in the body, were of two types, outlying or distal bones represented by the caudal vertebrae and central bones represented by the femur and the humerus. Huggins and Blocksom<sup>34</sup> have pointed out that in the rat the marrow of the outlying bones is characteristically of the hematopoietically inactive yellow type while that of the central bones is of the hematopoietically active red type. We assumed that by including representative bones of the two types we would be presented the opportunity of noting the reaction of both yellow and red marrow to anemia and to therapy. The marrow tissues of the two types of bones were found to be dissimilar both in the anemic state and after therapy and, therefore, require separate consideration and description.

Longitudinal sections of vertebrae from two portions of the tail, namely, vertebrae of the middle of the free portion of the tail and vertebrae immediately following the sacral vertebrae at the base of the tail, were prepared for histologic study of the marrow. The marrow tissue of the vertebrae of the two regions of the tail presented some points of difference. The marrow of the

distal caudal vertebrae, except for a few islands of hematopoietic tissue in the epiphyses and metaphyses, was of the yellow variety throughout. The marrow of these vertebrae resembled that described by Huggins and Bloksom<sup>24</sup> for normal adult rats, and it is apparent that in our animals the existence of a severe anemia had not produced active hematopoietic tissue in these vertebrae. The marrow of the distal caudal vertebrae showed no histologic changes in response to copper and iron therapy. The first caudal vertebra of anemic

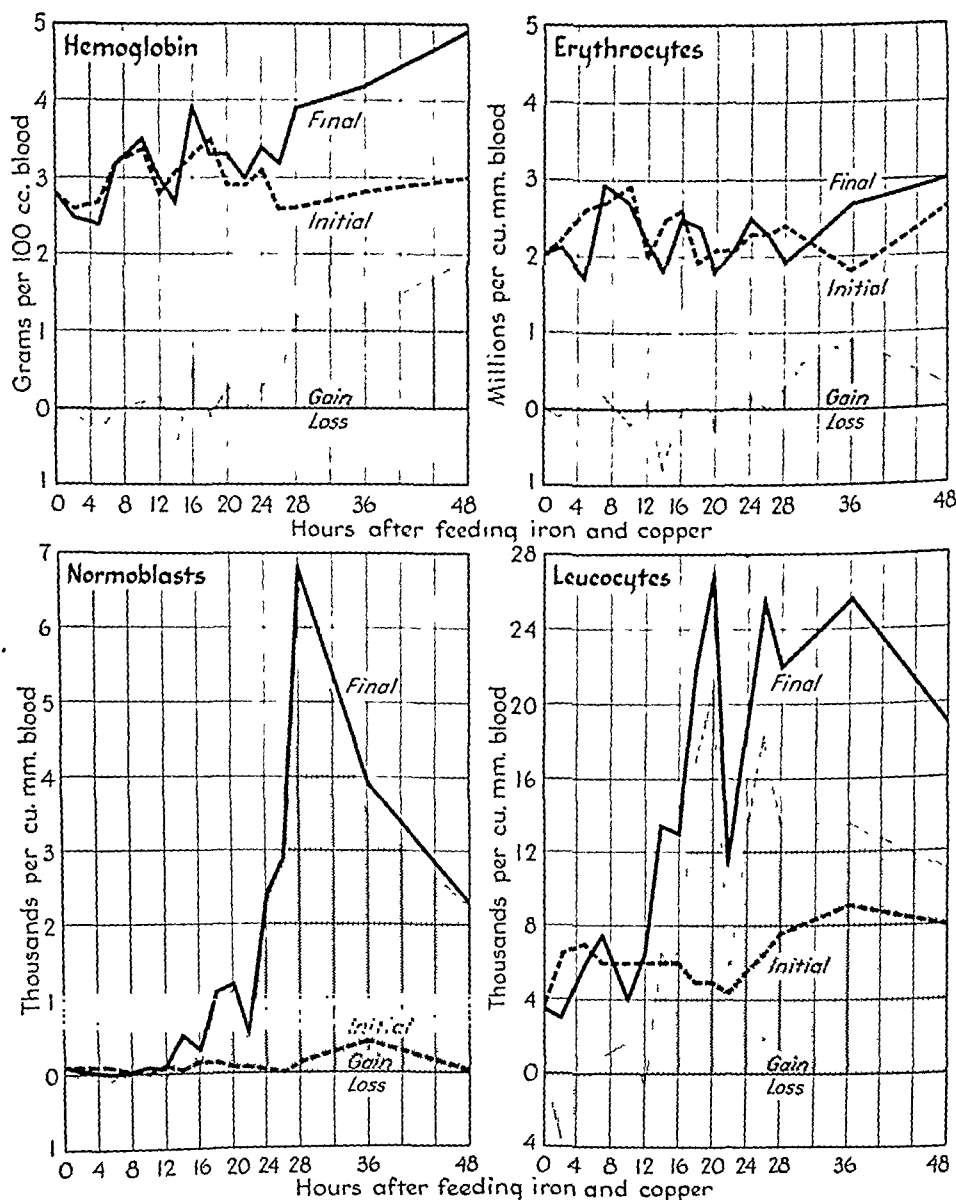


Fig. 1.—Effect on hemoglobin erythrocytes, normoblasts, and leucocytes of feeding a total of 0.5 mg. iron and 0.003 mg. copper per gram body weight.

animals possessed fairly abundant red marrow in both epiphysis and centrum. Fat cells were present in the marrow and were most abundant toward the posterior end of the vertebra. The first caudal vertebra possessed more red marrow and less yellow marrow than the second and third caudal vertebrae, and the third vertebra possessed less red and more yellow marrow than the second vertebra. The third caudal vertebra, therefore, possessed more yellow marrow than red marrow. It should be noted that for the second and third

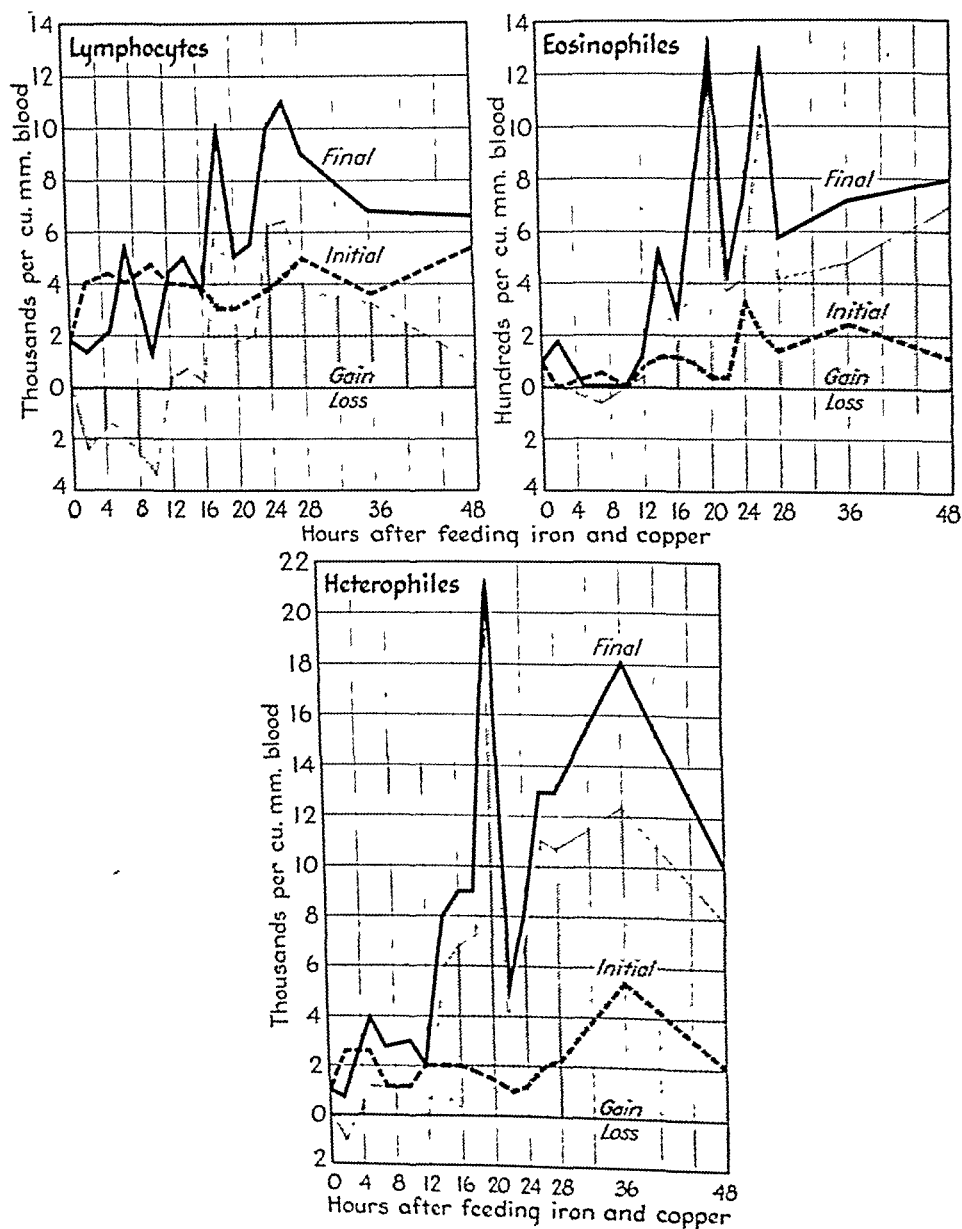


Fig. 2.—Effect on lymphocytes, eosinophiles, and heterophiles of feeding a total of 0.5 mg. iron and 0.003 mg. copper per gram body weight.

caudal vertebrae the red marrow was best developed and most active at the anterior end. The decrease in red marrow and increase in yellow marrow from the first to the third caudal vertebrae were anticipated because the first vertebra is essentially a central type of bone while the third caudal vertebra is essentially an outlying type of bone. Though histologic changes were found to occur in the red marrow of the first and to some extent in the second caudal vertebrae after therapy, the changes were not as extensive or as marked, though of the same nature, as those found to occur in the femur and humerus. For that reason further description of the marrow of the caudal vertebrae will not be given. Our observations indicate that the severe dietary anemia of our study did not cause the extension of red marrow into typical yellow marrow areas and that typical yellow marrow does not, for the 48-hour period of observation of this study, respond to copper and iron therapy.

The sections of the marrow removed from the femur before being placed in the fixative when stained with hematoxylin-eosin-azure II provided the best material for the identification and study of the various stages of hematopoiesis. However, the marrow removed from the marrow cavity before fixation did not include the marrow tissues at the periphery and epiphyseal ends of the marrow cavity and did not provide a complete picture of the marrow tissue. Longitudinal sections of the entire femur and also of the humerus, though not as sharply stained with the hematoxylin-eosin-azure II, provided excellent material for study of the cytology of red marrow as well as for the extent of the red marrow tissues. The marrow tissue of the femur and humerus, both in the anemic state and after therapy, were essentially similar as far as hematopoietic activity was concerned and were used to supplement each other in this study. The descriptions of the cytologic changes of the marrow to be given are based largely on the longitudinal sections of the entire femur and of the marrow removed from the femur before fixation. The smears and imprints were very useful in identifying hematopoietic cells and were used to supplement the sectioned material.

The histology and cytology of typical femur marrow of anemic rats of this experiment are illustrated in Fig. 3. It should be noted that the marrow tissue of anemic animals was of the hyperplastic red type throughout the entire extent of the femur and the humerus. Fat cells were very few in number and were most commonly found, scattered singly or in groups of a few cells, in the tissue at the periphery of the marrow of the central cavity and in the marrow of the epiphyses. The venous sinuses were prominent and well defined and the reticular lining cells forming their walls were continuous and distinct. There was no indication of a discontinuous wall of the venous sinuses in any of our sections in which the tissues were satisfactorily stained. The inter-sinusoidal tissue, or parenchyma, was sharply limited by the reticulo-endothelial walls of the venous sinuses and toward the central portion of the tissue were found the arteries and arterioles supplying it. The parenchymatous tissue was fairly cellular and compact though open enough to permit easy identification and study of the reticular cells forming its framework. Some of the reticular cells were in the process of mitosis and others, as shown by the lack

Figs. 3, 4, and 5.

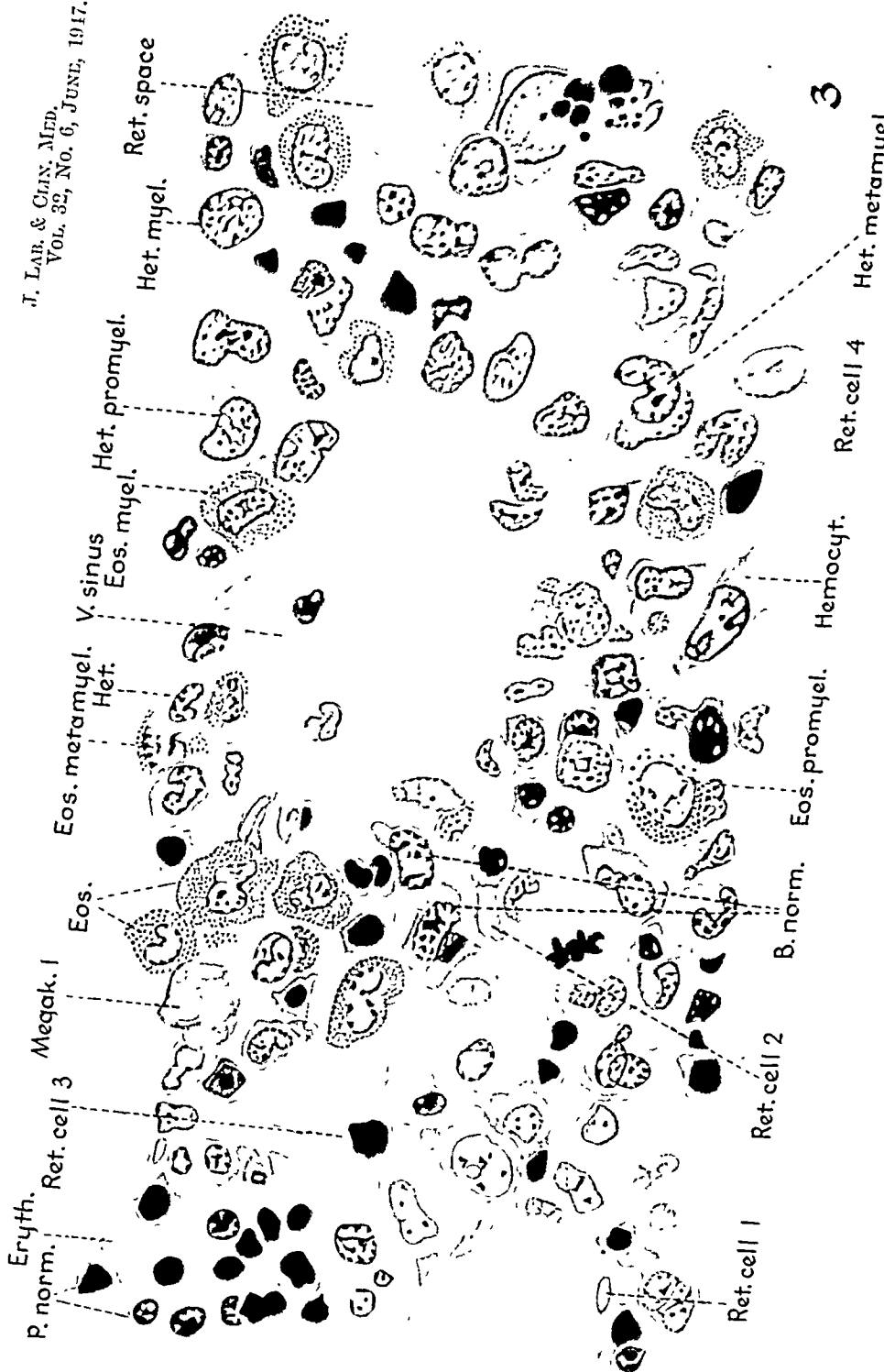
Sections of marrow of femur of untreated anemic rat (Fig. 3), anemic rat killed 22 hours after receiving 0.3 mg. iron and 0.22 mg. copper (Fig. 4), and anemic rat killed 48 hours after receiving 0.5 mg. iron and 0.26 mg. copper (Fig. 5).

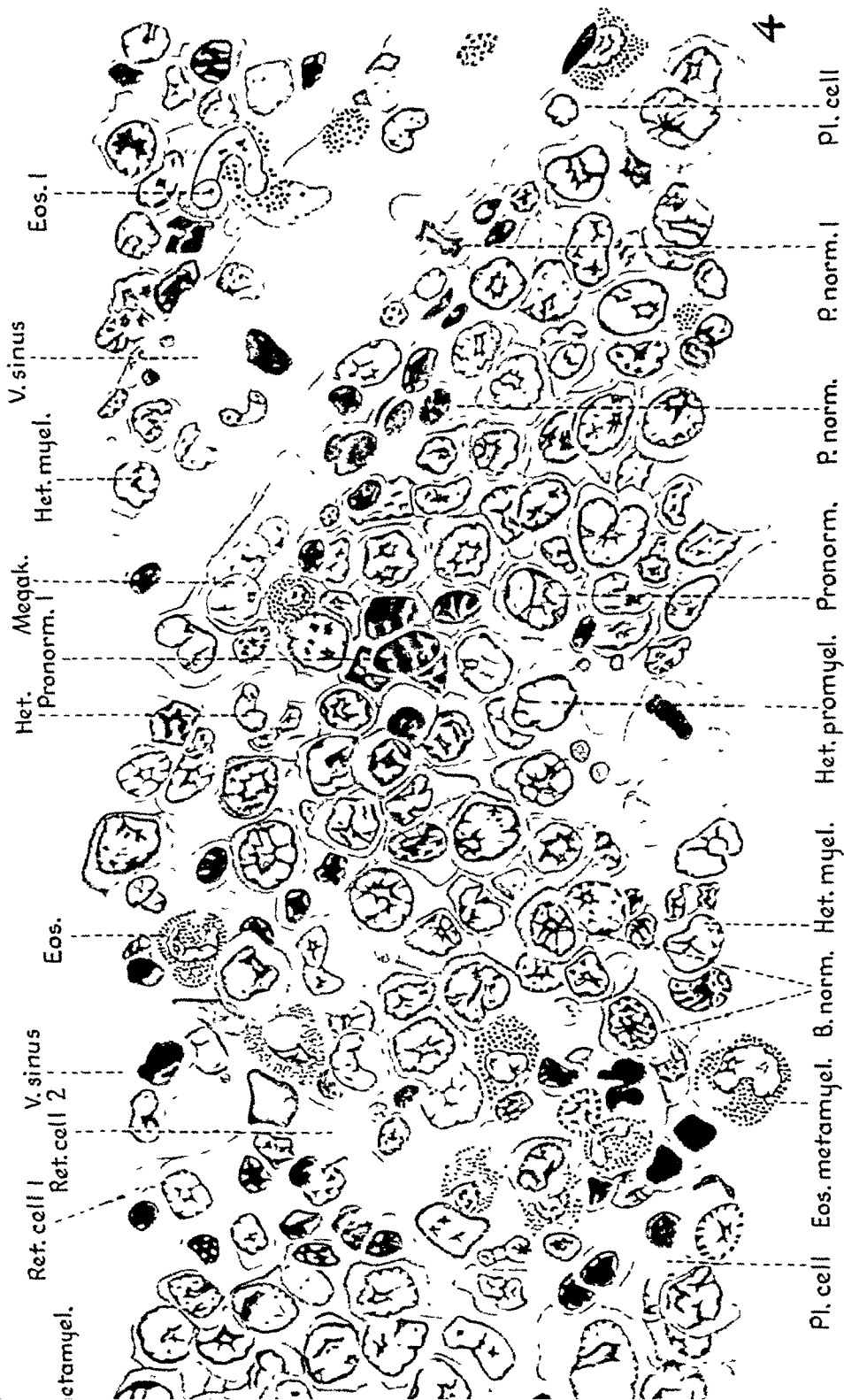
All tissues fixed in Zenker-formol and stained in hematoxylin-eosin-azure. Camera lucida drawings, X1100.

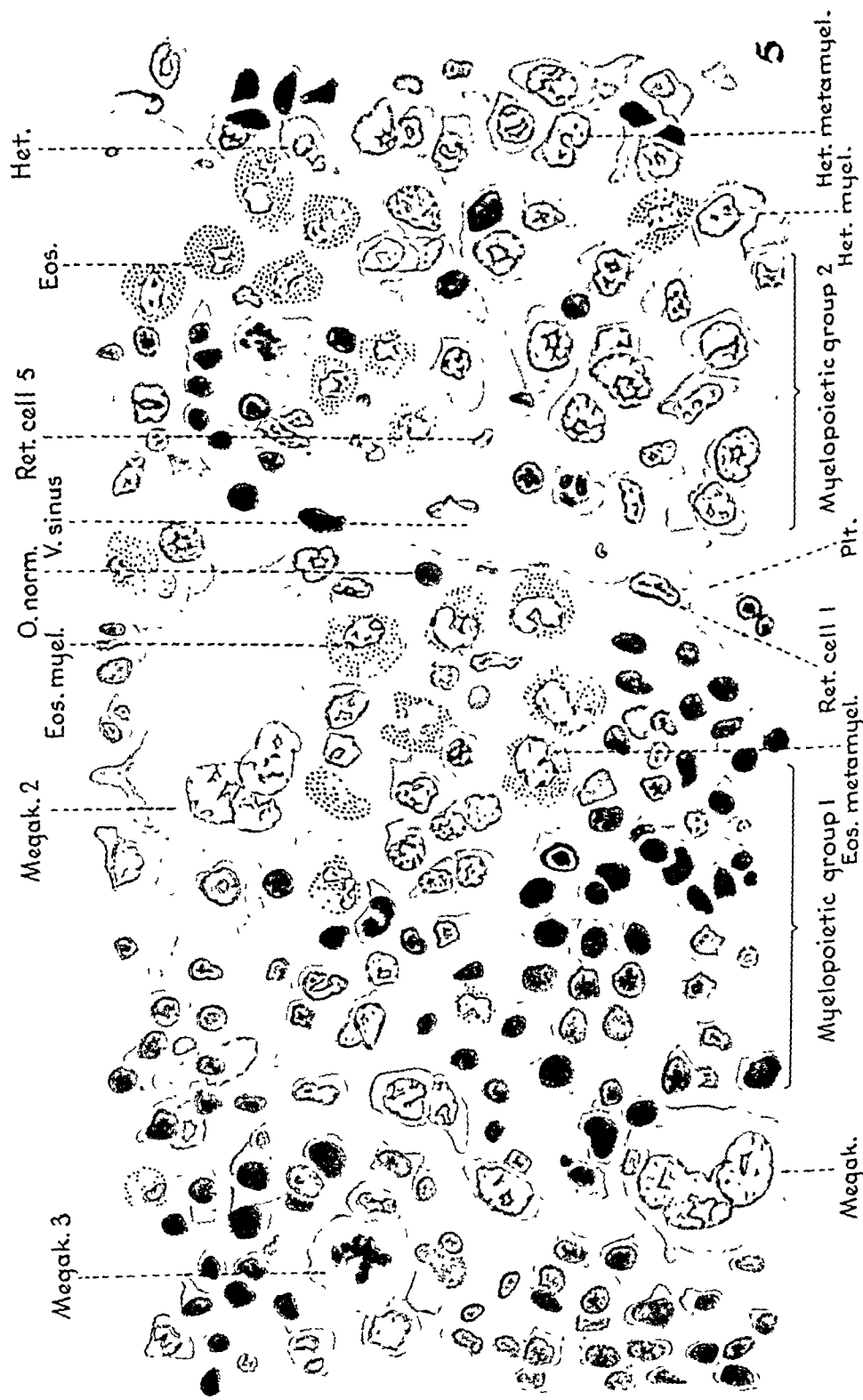
In all cases the abbreviations have the following meaning: *B.*, basophile; *P.*, polychromatophile; *Pl.*, plasma; *O.*, orthochromatophile; *Eos.*, eosinophile; *Eryth.*, erythrocyte; *Hemacut.*, hemocytoblast; *Ret.*, reticophile; *Meqak.*, megakaryocyte; *myel.*, myelocyte; *metamyel.*, metamyelocyte; *norm.*, normoblast; *Plt.*, platelet; *P.enorm.*, pronormoblast; *Ret.*, reticular; *V.*, venous.

Eosinophile (*Eos.*, 4, Fig. 4) and polychromatophile normoblast (*P. norm.*, 4, Fig. 4) entering venous sinus; pronormoblast in mitosis (*Meqak.*, 4, Fig. 4); megakaryocytes in mitosis (*Meqak.*, 4, Fig. 5), showing platelet formation (*Meqak.*, 2, Fig. 5), and in degeneration (*Myelopofietic group 2*, Fig. 5); group of normoblasts in homoplastic development (*Myelopofietic group 1*, Figs. 3, 4, and 5) and heterophiles in homoplastic formation (Figs. 3 and 4), stroma reticular cell in mitosis (*Ret. cell 1*, Figs. 3, 4, and 5), reticular cells of stroma (*Ret. cell 2*, stroma reticular cell bordering on lining cell of venous sinus (*Ret. cell 3*, Fig. 3), reticular cell transforming into hemocytoblast (*Ret. cell 3*, Fig. 3), and









of long processes, basophilia of the cytoplasm, and nucleus with coarse chromatin and distinct chromatin nucleoli, were in the process of changing into hemocytoblasts. The reticular cells at the margin of the intersinusoidal tissue sent processes to the reticular lining cells of the venous sinuses and contributed to the formation of the wall of those vessels. Other reticular cells sent their processes to the walls of the arterioles and arteries located in the stroma. The hematopoietic tissues located in the reticular spaces of the stroma were divided into separate and fairly well-defined groups of differentiating erythrocytes, eosinophiles, and heterophiles. The tendency of the hematopoietic cells to separate into groups of similar cells was best seen in areas of longitudinal sections through the intervacular tissue and the neighboring venous sinuses.

The primary hematopoietic process of the bone marrow of anemic animals was of the homoplastic type. Orthochromatophile and polychromatophile normoblasts, the latter the most numerous, formed the greater part of the nests of erythropoietic cells. It should be emphasized that the cytoplasm of the orthochromatophile normoblasts, as shown in Fig. was not acidophilic as in normal bone marrow but more or less strongly basophilic and stained with the azure stain instead of the eosin stain. They were readily identified, however, by their deeply stained pyknotic nuclei. The basophile character of the cytoplasm of the orthochromatophile normoblasts of the marrow of our anemic animals was expected because many of the erythrocytes of the circulating blood lacked hemoglobin and possessed basophilic or faintly acidophilic cytoplasm. The polychromatophile normoblasts likewise were identified by their nuclei, of coarse, radially arranged and deeply stained chromatin masses and not by the cytoplasm which was basophilic rather than polychromatophilic. Basophile normoblasts were few in number. They were characterized by deeply stained basophilic cytoplasm and a nucleus possessing fairly large chromatin nucleoli and coarse chromatin granules placed on the inner surface of the nuclear membrane. Pronormoblasts were encountered fairly frequently in the nests of erythropoietic cells and differed from the basophile normoblasts in possessing more prominent chromatin nucleoli, finer chromatin granules on the nuclear membrane, and a more irregular cell outline. Mitoses of the erythropoietic cells were very infrequent and it appears that the low erythrocyte count of the anemic animals was the result of arrested cell multiplication and differentiation.

Nests of eosinophilic cells, being almost as numerous as nests of heterophilic cells, were more numerous than the number of eosinophile leucocytes of the circulating blood indicated should have been present. The granules of the developing eosinophilic cells were large and brilliantly stained by the eosin dye. Mature eosinophiles were identified by the presence of an abundance of eosinophilic granules, a faintly acidophilic cytoplasm, and a nucleus more or less deeply indented to form two nuclear lobes. The chromatin of the nucleus of the eosinophiles included small and medium-sized granules in the interior and granules of irregular size placed on the inner surface of the nuclear membrane. The eosinophile metamyelocytes also possessed numerous granules, faintly acidophilic cytoplasm, and a nucleus only slightly indented or perforated at

the center or toward one side. Formation of the eosinophile metamyelocytes from myelocytes was either by indentation or perforation of the nucleus, the latter method of formation occurring most frequently. The eosinophile myelocytes formed the most common cell of the eosinophile nests. They possessed a slightly basophilic cytoplasm, fairly numerous granules, and a spherical nucleus with chromatin nucleoli and numerous fairly fine chromatin granules on the nuclear membrane. Eosinophile promyelocytes, though rarely encountered, were readily identified by their basophilic cytoplasm, reduced number of granules, and a large spherical nucleus. The chromatin of the nucleus of the promyelocyte included fairly large chromatin nucleoli and fine chromatin granules in the interior and on the nuclear membrane. Hemocytoblasts, or myeloblasts, of the eosinophile nests were not numerous but were more numerous than the promyelocytes. They resembled the pronormoblast in shape, basophile staining of the cytoplasm and in the presence of large irregular chromatin nucleoli in the nucleus. They differed from the pronormoblasts in that the chromatin granules on the nuclear membrane were finer and more numerous than those found on the membrane of the pronormoblast. Mitoses of the myelocytes were very infrequent, and it appears that for the eosinophile series, as for the erythrocyte series, cell multiplication and differentiation was suppressed by the anemia.

The cells of the heterophile series of leucocytes also occurred in irregular nests but were the most difficult to identify because the cytoplasm of even the most mature cells of the series was faintly basophilic and because specific granules were not stained or when stained were so small as to be difficult to see. However, it was possible to identify the various stages of differentiation of the heterophiles by comparing their nuclei with the nuclei of the various differentiation stages of the eosinophiles. Since the structural features of the nuclei of the differentiation stages of the heterophiles are like those of the eosinophiles, descriptions of them will not be repeated here. The heterophile promyelocytes were very difficult to identify because of their close resemblance to hemocytoblasts and because of the absence of specific granules. Myelocytes were fairly numerous and differentiation into metamyelocytes was by nuclear perforation or indentation and, as for eosinophiles, most frequently by the former method. Late heterophile metamyelocytes possessed a slender bandlike nucleus while mature heterophiles possessed two or more nuclear lobes. Mitoses of the myelocytes of this series were very infrequent and multiplication and differentiation of the cells of the heterophile series were also suppressed by the anemia.

Basophiles, or mast cells, were encountered very infrequently in the marrow of any of our animals. Plasma cells were found in the marrow of our anemic animals and were usually irregularly distributed through the tissue. Large-sized heterophiles and eosinophiles, most frequently encountered in the myelocyte and metamyelocyte stages, were found in the marrow of the untreated animals. Megakaryocytes showing platelet formation were not numerous. Naked nuclei of degenerating megakaryocytes were also present. An occasional megakaryocyte

was phagocytic, the phagocytosed cells most frequently being normoblasts readily identified by their small, spherical pyknotic nuclei.

The histologic and cytologic changes occurring in the marrow of anemic animals following the feeding of copper and iron supplements can best be described from marrows which had reached the point of greatest response to the therapy. One of the reactions to therapy was the appearance of mitoses in the cells of erythrocyte, eosinophile, and heterophile series. A second and unusual reaction to the therapy was the appearance of large numbers of pronormoblasts and heteroplastic erythropoiesis. The characteristics of a bone marrow showing that reaction in a high degree of development is illustrated in Fig. 4. The marrow illustrated was from an animal killed 22 hours after supplement feeding. Other marrows, namely, of individuals killed at 18, 20, 24, and 26 hours after feeding, showed approximately the same degree of development of heteroplastic erythropoiesis. The occurrence of well-developed erythropoiesis of that type for that fairly long period of time can be accounted for by individual variation in response to therapeutic treatment. The variation observed can be correlated with a similar variation in the values of normoblasts and leucocytes of the circulating blood shown for the same period of time in the graphs of Figs. 1 and 2. It should be noted that the marrow of other animals killed during this period of time also showed heteroplastic erythropoiesis but that the heteroplastic tissue did not occupy as great an area of the marrow.

The marrow at the height of response to therapy was characterized by greater cellularity and compact appearance of the intersinusoidal parenchyma. Venous sinuses, as for anemic animals, were well developed and prominent and their walls well defined. Arteries were located in the central portion of the intersinusoidal tissue area, and in general the vascular system and the intersinusoidal stroma tissue were the same as those found in the marrow of anemic animals. The intersinusoidal hematopoietic tissues were characterized by the presence in the central portion of the area and, therefore, surrounding the arteries and arterioles of a large number of pronormoblasts. Many of the pronormoblasts were in mitosis or were in preparation for mitosis. To either side of the mass of pronormoblasts, between them and the reticular lining cells of the wall of the venous sinuses, were found megakaryocytes and groups of erythrocytes, eosinophiles, and heterophiles in the later stages of differentiation. It was clearly evident in many preparations that the cells of this region were compressed by the new growth occurring in the central region of the intersinusoidal area. Numerous instances of normoblasts and granulocytes passing through the wall of reticular lining cells into the sinuses were found in these preparations. The constricted character of the cells passing through the membrane emphasizes the fact, also readily seen in the sections, that the reticulo-endothelial wall of the venous sinuses is a continuous one and that cells pass between the margins of the reticular lining cells. We have concluded that growth pressure within the intersinusoidal areas of our material assists in the migration of normoblasts from the intersinusoidal tissue into the venous sinuses.

The marrows of animals killed at earlier hours after feeding of copper and iron were studied in an effort to determine the origin of the pronormoblasts

which appear in such large numbers during the period of 18 to 26 hours after supplement feeding. It should be emphasized again that the marrow of anemic animals was hyperplastic but that cell multiplication and differentiation was depressed. It should also be pointed out that the vascular system of the marrow of anemic animals, of animals killed a few hours after supplement feeding, and of animals killed at the highest point of heteroplastic hematopoiesis were identical. The pronormoblasts appeared in the intersinusoidal areas and not in the vascular channels. It was noted earlier that instances of reticular cells of the intersinusoidal areas transforming into hemocytoblasts were found in our preparations of the marrow of anemic animals. Instances of such transformation were also found in the marrow of animals killed a few hours after therapy but instances of transformation were not very numerous. Transformation of reticular cells into hemocytoblasts does not satisfactorily account for the formation of such large numbers of hemocytoblasts. However, marrows of animals killed at 8 to 14 hours show many of the hemocytoblasts and pronormoblasts present to be in mitosis, and it appears that the greater number of pronormoblasts were formed in situ by multiplication of those cells and to a lesser degree by transformation of reticular cells into hemocytoblasts.

The marrows of animals killed at 36 and 48 hours after feeding of the iron and copper supplements illustrated in Fig. 5, show the usual arrangement of the venous sinuses and the intersinusoidal tissues. However, pronormoblasts were present in small numbers only, and it is evident that the heteroplastic erythropoiesis had disappeared as the result of the transformation of the pronormoblasts into the later erythropoietic stages. As a result, homoplastic hemopoiesis had been restored. Polychromatophilic and orthochromatophilic normoblasts, many of the former in mitosis, were very numerous. When the marrow of anemic animals and animals killed 48 hours after therapy were compared, it was found that the erythropoietic tissues were more extensive in the latter than in the former marrows. Nests of eosinophiles and heterophiles in homoplastic development were also present. Small megakaryocytes were fairly numerous and large well-developed megakaryocytes showed active platelet formation. Plasma cells and large eosinophiles and heterophiles were present but few in number. All features of the marrow of animals killed after 36 and 48 hours point to a normal condition of very active homoplastic blood cell formation.

#### DISCUSSION

The present study on dietary anemia of the rat produced by feeding milk alone confirms earlier work in showing that regeneration of the blood is initiated by the addition of copper and iron to the diet. It supports the work of Hamre and Miller<sup>19, 20</sup> on the spleen and Fitz-Hugh and associates<sup>27</sup> on the bone marrow, in showing that the blood-forming organs respond to copper and iron therapy by increased hematopoietic activity. It further shows that the increased cellularity of the bone marrow of recovering anemic rats fed copper and iron noted but not identified by Fitz-Hugh and associates was due to an increase of homoplastic hematopoietic tissue. The material of this study definitely shows that accelerated homoplastic erythropoiesis is established as early as 36

hours after the initial copper and iron administration. It also shows that the first hematopoietic response to copper and iron therapy is the development of heteroplastic erythropoiesis. The heteroplastic erythropoiesis reaches its highest state of development some time during the period of 18 to 26 hours after adding copper and iron to the milk diet. The early heteroplastic erythropoiesis supplies increased erythropoietic tissue for the homoplastic erythropoiesis established 36 to 48 hours after the supplement feeding.

That the bone marrow of anemic animals does respond rapidly and strongly to effective therapeutic agents has also been indicated by other investigators. Doan and co-workers<sup>35</sup> state that the hematopoietic response of the hypoplastic marrow of the radius and the ulna of starved pigeons occurs only 18 hours after feeding is resumed. At that time differentiation of new fat cells begins and signs of reactivation of erythropoiesis appear. They point out that by 50 hours after feeding a normal appearance of the marrow has been established. Jordan and Johnson<sup>36</sup> also investigated the radial marrow of starved pigeons and state that at 28 hours after feeding is resumed hematopoiesis is established and that after that time the only feature of importance respecting hematopoiesis is that it becomes more intense and widespread. The work of McDonald<sup>37</sup> on the hypoplastic marrow of starved pigeons agrees with the observations of the previously mentioned two groups of investigators as far as the time of appearance of increased erythropoietic activity is concerned. Kandel and LeRoy<sup>38</sup> have indicated that in pernicious anemia in man fourteen and a half hours after liver therapy, the megaloblastic character of the sternal marrow has largely disappeared and has been replaced by an increased amount of normoblastic tissue. Bock and Malamos<sup>39</sup> reporting on their own observations and quoting observations of other European workers also point out that there is a rapid transformation of the marrow of patients with pernicious anemia after liver treatment and state that at 48 hours after first treatment megaloblastic cells are difficult to find while the early stages of the erythro-normoblastic type of cells are very numerous. It is obvious that the initial response of the bone marrow of the patients examined by the last group of authors occurred at some earlier hour of the first two days of recovery. Other papers pointing to the time of response of the bone marrow of animals to therapeutic treatment may exist in the literature on anemia but those cited previously in this paper support our view that a "critical period" of erythropoiesis occurs during the first few hours after effective therapy of anemia. For our anemic rats the critical period of recovery extended from 18 to 26 hours after the initial feeding of copper and iron and was characterized by a high degree of development of heteroplastic erythropoiesis. The fact that the height of heteroplastic erythropoiesis did not occur at some one hour of that period for all animals can be explained by the commonly accepted fact of existence of individual differences in the state of a disease and individual differences in reaction to therapy.

The studies of the changes of the blood values of rats recovering from anemia carried out in this investigation show that increases of values for normoblasts, eosinophiles, and heterophiles occur during a period of time which coincides with the critical period of hematopoiesis of the marrow. This period



for those blood cells extends from 12 to 26 hours after therapy. The coinciding of the periods of change of blood values and of the histology of the bone marrow suggest that the two are related and our material shows that the development of heteroplastic erythropoietic tissue is accompanied by a discharge of those blood cells into the venous sinuses. Hamre and Miller,<sup>39</sup> in studying the regeneration of the blood elements of anemic rats following copper and iron feeding, point out that for normoblasts and total number of leucocytes, chiefly heterophiles, the highest values occur on the second and the fifth or sixth day of recovery. Our material suggests that the high values for the second day are the result of the development of heteroplastic erythropoietic tissue in the marrow and the coincident discharge of cells present at the time treatment was begun. We suggest that the second period of high values occurring on the fifth or sixth day of recovery may mark the beginning discharge of a new generation of cells produced from the heteroplastic tissue of the critical period of hematopoiesis. Investigation of the marrow of animals in the second to the fifth day of recovery is needed to establish the significance of the second period of high values of normoblasts and leucocytes.

The marrows of the animals of this investigation, since they possessed large quantities of heteroplastic erythropoietic tissue, form material which bears on the controversial problem of the place within the marrow in which erythrocytes originate and differentiate. The literature bearing on this problem has been reviewed by Doan and co-workers<sup>35</sup> and Sabin and Miller,<sup>40</sup> and a review of the literature will not be presented here. Two views have been advanced in the literature on hematopoiesis, the first view that granulocytes develop extravascularly while erythrocytes develop intravascularly and the second view that both granulocytes and erythrocytes develop extravascularly. Differences of opinion on the place of differentiation of blood-cells in the bone marrow, therefore, center primarily about the place of differentiation of erythrocytes; it should be noted that the differences of opinion apply to erythropoiesis in the bone marrow of adult animals since the work of recent authors, Maximow<sup>41</sup> and Bloom and Bartelmez<sup>42</sup> on erythropoiesis in mammalian and human embryos and Dantchakoff<sup>43</sup> and Sabin<sup>44</sup> on avian embryos, agree in describing erythropoiesis as occurring intravascularly. The first view of erythropoiesis, therefore, holds that the embryonic type of intravascular erythropoiesis is continued in the marrow of the adult animal, while the second view holds that embryonic intravascular erythropoiesis during development is lost and is replaced by a new extravascular erythropoiesis in the bone marrow of the adult animal.

The more recent interpretations of the view that erythropoiesis occurs intravascularly in the marrow of adult animals is to a great extent based on the studies of Doan and co-workers<sup>35</sup> on the regeneration of the hypoplastic marrow of starved pigeons after normal feeding was resumed. They concluded that granulocytes are produced in the intervacular stroma but that erythrocytes are produced from the endothelium of hematopoietic capillaries, called intersinusoidal capillaries, originally described by Doan.<sup>45</sup> After maturing within the capillaries, the erythrocytes were described as being carried directly into the circulation and the capillaries as opening to the circulation

and temporarily ceasing erythropoiesis. Jordan and Johnson<sup>36</sup> and McDonald<sup>37</sup> after repeating the studies of these authors<sup>33, 45</sup> doubt or deny the existence of intersinusoidal capillaries in the number or type described and suggest that many of the spaces so labeled are stromal reticular spaces. The latter authors<sup>36, 37</sup> agree that granulocytes take origin in stromal spaces not open to the circulation. They also hold that cells of the erythrocyte series may mature within vascular channels but that the stem cells take origin from the extravascular stroma and then migrate into the vessels for maturation. McDonald also states that some of the erythropoietic cells of the vessels may take origin directly from the endothelium of the vessels without passing through the myeloblast stage. The recent literature on erythropoiesis in the bone marrow of birds indicates that differentiation, and possibly also origin, of erythrocytes occurs intravascularly.

Doan and co-workers<sup>35</sup> have also reported that intersinusoidal capillaries, similar to those described for the marrow of the pigeon, occur in the bone marrow of rabbits. They, therefore, reported erythropoiesis in the rabbit to be intravascular. Peabody<sup>46</sup> claimed to have seen intersinusoidal capillaries in the marrow of man and described erythropoiesis as occurring within them. However, the generally accepted view of hematopoiesis in the marrow of mammals, as noted by Bloom,<sup>47</sup> is that both leucopoiesis and erythropoiesis are extravascular. The generally accepted view of extravascular hematopoiesis is based largely on the clear and detailed descriptions of the process and the histology of the bone marrow of mammals given by Maximow.<sup>45</sup> The work of Drinker and associates<sup>49</sup> and Bunting<sup>50</sup> on the marrow of dogs, cats, and rabbits does not support the idea of the existence of intersinusoidal erythrocytogenic vessels but does support the view that in mammals granulocyte and erythrocyte formation is extravascular.

The review of recent investigations of hematopoiesis in the marrow of birds and mammals gives the impression that granulocytopoiesis is extravascular in both but that they may differ with respect to erythropoiesis. We do not deny the possibility of intravascular erythropoiesis in birds but on the basis of the evidences presented in the literature conclude that it may occur. On the other hand, we feel that evidences presented in the literature point to extravascular erythropoiesis in mammals. Our material on the marrow of the rat, in that it did not show erythrocytogenic vessels comparable to the so-called intersinusoidal capillaries or a change in the pattern of the vascular system during the initiation and development of erythropoiesis, supports that view. We feel that the appearance of new heteroplastic erythropoietic tissue in the parenchyma of the marrow, absence of masses of differentiating erythrocytes from the vascular channels, and the lack of hematopoietic activity on the part of the endothelial lining of the vessels lend strong support to the view that in adult mammals all erythropoiesis is extravascular and localized in the intervacular areas of the marrow.

The heteroplastic erythropoietic marrows of the rats of this study, as pointed out in an earlier section of this paper, supply evidences on the manner of discharge of the inactive erythrocytes from the intersinusoidal areas into the

circulating blood. Explanations of the manner in which erythrocytes are so discharged would differ for instances of intravascular and extravascular erythropoiesis. In the former instance, as pointed out by Doan co-workers,<sup>35</sup> Jordan and Johnson,<sup>36</sup> and McDonald,<sup>37</sup> the erythrocytogenic vessels are continuous with the nonerythrocytogenic vessels and the mature erythrocytes are carried directly into the actively circulating blood of the latter. Explanations of the stimulation of release of the mature cells are not offered. In instances of extravascular erythropoiesis, the endothelial lining cells of the sinuses serve as barriers through which the red blood cells must pass to reach the circulating blood. It is on the possible manner in which this is accomplished that our material yields information.

Drinker and associates<sup>40</sup> have reviewed the literature dealing with the problem of the manner of discharge of red blood cells through the endothelial membrane. They suggest that the endothelial membrane of the sinuses is a continuous one and that groups of erythrocytes expand by growth onto the membrane and due to slight differences in pressure inside and outside the vessel are carried through the membrane. Maximow<sup>48</sup> also held that the membrane is continuous about all of the vessels of the marrow but that over masses of erythrocytes it may temporarily open or rupture to expose the cells to the circulating blood. The plasma passing through the temporary opening loosens the cells and carries them into the circulation. After discharge of the cells the thin endothelial lining membrane closes and the wall of the vessel regains continuity. Key<sup>51</sup> has suggested that the maturation of the erythrocytes is accompanied by a decrease in their cohesiveness and that, as a result, the red blood cells are more readily washed out of the stroma spaces through openings in the endothelial wall into the blood stream. Bunting<sup>50</sup> has suggested that growth pressure may be a factor in causing migration of the red blood cells, though he does not state definitely whether or not he believes the endothelial membrane to be continuous or perforated.

The endothelial membrane of the venous sinuses of our material, as was pointed out previously, is continuous and distinct. Numerous instances of granulocytes and normoblasts passing through the endothelial membrane were found in our material and it appears to us that constriction of both granulocytes and normoblasts at the membrane is significant. We have concluded that the cells pass through the membrane between the margins of the endothelial lining cells and we further have concluded that the intrastromal growth of large quantities of erythropoietic tissue forced the cells through the membrane into the sinuses. We feel that growth pressure is one of the factors, though undoubtedly not the only factor, which brings cells of the erythrocyte series from the intervaseular tissue into the blood vessels.

The subject of the origin of the blood cells forms such an extensive mass of publications that it cannot be reviewed here. However, Downey<sup>52</sup> and Cunningham and associates<sup>53</sup> have reviewed the literature and the reader is referred to those publications for detailed information on the subject. It should be noted that the latter authors and Doan and co-workers<sup>35</sup> have advanced the theory that in the bone marrow of adult animals the erythrocyte series takes

origin from the endothelial lining cells of hematopoietic capillaries. Many authors hold that the endothelial lining cells do not possess the potentiality of differentiating into hematopoietic cells and our material supports that view. In our series of animals, the bone marrow was stimulated by copper and iron therapy to produce large quantities of heteroplastic erythropoietic tissue, including large numbers of pronormoblasts. The new tissue was located extravascularly and in none of our preparations did the endothelial lining cells of the sinuses show a change in form or exhibit tendencies toward differentiation of any type.

As was pointed out in the section on results of this study, a special effort was made to determine the source and origin of the pronormoblasts found in such large numbers in our preparations. We did find instances of the reticular cells of the intervascular stroma transforming into hemocytoblasts. Instances of such transformation were not numerous enough to account for the numbers of pronormoblasts produced. However, we did find that after therapy mitosis of the hemocytoblasts was greatly increased. We have concluded that the greater number of pronormoblasts arose by multiplication of hemocytoblasts and pronormoblasts already present at the time therapy was begun or of hemocytoblasts produced by transformation of stromal reticular cells after copper and iron feeding. In none of our preparations were we able to discover cells comparable to the "primitive cell" described by Sabin and associates<sup>54</sup> as the progenitor of granulocytes. Myeloblasts of the type described by Downey,<sup>55</sup> or hemocytoblasts as described by Block,<sup>56</sup> were present in the smears and sections of the bone marrow of our recovering animals. We conclude that they formed the stem cells for granulocytopoiesis and of the pronormoblasts of our preparations.

#### SUMMARY AND CONCLUSIONS

1. The blood values and bone marrows of sixty anemic rats killed at hourly intervals during the initial 48-hour recovery period after copper and iron treatment were observed in this investigation.

2. Hemoglobin and erythrocytes showed an increase in value beginning at 26 hours after treatment, and a slight but constant increase was continued from that hour to the end of the 48-hour observation period.

3. Normoblasts, eosinophiles, and heterophiles increased in value beginning at 12 hours after treatment, and a period of high and moderately high values extended from that hour to 26 or 28 hours after therapy, this being the period of first discharge of those cells from the bone marrow and the irregularity of values being due to variation in time and extent of response to therapy. Following that period of increase, the values for the various leucocytes continued higher than normal to the end of the period of observation.

4. The first histologic response of the bone marrow to copper and iron treatment was the development of heteroplastic erythropoietic tissue, including numerous pronormoblasts, and the highest degree of development of the tissue was found in the marrows of animals killed 18 to 26 hours after therapy. This period we have called the "critical period" of hematopoiesis of recovery.

5. The period of first great increase in numbers of normoblasts, eosinophiles and heterophiles coincided with critical period of hematopoiesis of the bone marrow and our histologic preparations showed a discharge of those cells into the blood stream by the bone marrow to occur during that period.

6. Erythropoiesis after the critical period was characterized by the disappearance of the heteroplastic process and the development of the homoplastic process, which was continued in increased volume to the end of our period of observation.

7. Erythropoiesis, including the heteroplastic erythropoiesis, and leucopoiesis developed extravascularly in the intervaseular stroma; our observations, therefore, support the view that all leucopoiesis and erythropoiesis in the marrow of mammals is extravascular.

8. The lining membrane of the sinuses of our preparations of the marrows was continuous and distinct and there was no rupture or destruction of the membrane during discharge of cells from the intersinusoidal tissues into the sinuses.

9. The development of large quantities of new hematopoietic tissue in the intervaseular stroma, and the coincident discharge of cells into the sinuses, suggests that one of the factors concerned in the discharge of cells by the marrow is that of growth pressure within the intervaseular stroma.

10. Origin of the pronormoblasts of the new erythropoietic tissue in our preparations was not, as suggested by some authors, from the vascular endothelium but from stromal reticular cells and by multiplication of hemoctoblasts and pronormoblasts. "Primitive cell" progenitors of granulocytes, as described by Sabin and associates, was not recognized in our preparations, and we have concluded that the hemoctoblasts, or myeloblasts, quite numerous in our preparations, were the progenitors of the granulocytes.

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#### REFERENCES

1. Waddell, J., Steenbock, H., Elvehjem, C. A., and Hart, E. B.: Iron in Nutrition. V. The Availability of the Rat for Studies in Anemia, *J. Biol. Chem.* 77: 769-775, 1928.
2. Waddell, J., Elvehjem, C. A., Steenbock, H., and Hart, E. B.: Iron in Nutrition. VI. Iron Salts and Iron-Containing Ash Extracts in the Correction of Anemia, *J. Biol. Chem.* 77: 777-795, 1928.
3. Hart, E. B., Steenbock, H., Waddell, J., and Elvehjem, C. A.: Iron in Nutrition. VII. Copper as a Supplement to Iron for Hemoglobin Building in the Rat, *J. Biol. Chem.* 77: 797-812, 1928.
4. Waddell, J., Steenbock, H., and Hart, E. B.: Iron in Nutrition. VIII. The Ineffectiveness of High Doses of Iron in Curing Anemia in the Rat, *J. Biol. Chem.* 83: 243-250, 1929.
5. Waddell, J., Steenbock, H., Elvehjem, C. A., and Hart, E. B.: Iron in Nutrition. IX. Further Proof That the Anemia Produced on Diets on Milk and Iron is Due to Deficiency of Copper, *J. Biol. Chem.* 83: 251-260, 1929.
6. Waddell, J., Steenbock, H., and Hart, E. B.: Iron in Nutrition. X. The Specificity of Copper as a Supplement to Iron in the Cure of Nutritional Anemia, *J. Biol. Chem.* 84: 115-130, 1929.
7. Elvehjem, C. A., and Sherman, W. C.: The Action of Copper in Iron Metabolism, *J. Biol. Chem.* 98: 309-319, 1932.
8. Schultze, M. O., and Elvehjem, C. A.: The Relation of Iron and Copper to the Reticulo-cyte Response in Anemic Rats, *J. Biol. Chem.* 102: 357-371, 1933.

9. Hart, E. B., Elvehjem, C. A., and Kohler, G. O.: Does Liver Supply Factors in Addition to Iron and Copper for Hemoglobin Regeneration in Nutritional Anemia? *J. Exper. Med.* 66: 145-150, 1937.
10. Beard, H. H., and Myers, V. C.: Studies in the Nutritional Anemia of the Rat. I. Influence of Iron Upon Blood Regeneration, *J. Biol. Chem.* 94: 71-88, 1931.
11. Myers, V. C., and Beard, H. H.: Studies in the Nutritional Anemia of the Rat. II. Influence of Iron Plus Supplements of Other Inorganic Elements Upon Blood Regeneration, *J. Biol. Chem.* 94: 89-110, 1931.
12. Beard, H. H., Rafferty, C., and Myers, V. C.: Studies in the Nutritional Anemia of the Rat. III. The Prevention of Anemia by Means of Inorganic Elements, *J. Biol. Chem.* 94: 111-115, 1931.
13. Myers, V. C., Beard, H. H., and Barnes, B. O.: Studies in the Nutritional Anemia of the Rat. IV. The Production of Hemoglobinemia and Polycythemia in Normal Animals by Means of Inorganic Elements, *J. Biol. Chem.* 94: 117-122, 1931.
14. Beard, H. H., Baker, R. W., and Myers, V. C.: Studies in the Nutritional Anemia of the Rat. V. The Action of Iron and Iron Supplemented With Other Elements Upon the Daily Reticulocyte, Erythrocyte, and Hemoglobin Response, *J. Biol. Chem.* 94: 123-134, 1931.
15. Beard, H. H.: Studies in the Nutritional Anemia of the Rat. VI. The Effect of Inorganic Elements Upon the Rate of Blood Regeneration and Growth, *J. Biol. Chem.* 94: 135-146, 1931.
16. Eveleth, M. W., Bing, F. C., and Myers, V. C.: Studies in the Nutritional Anemia of the Rat. VII. Influence of Parenterally Administered Iron, *J. Biol. Chem.* 101: 359-368, 1933.
17. Bing, F. C., Saurwein, E. M., and Myers, V. C.: Studies in the Nutritional Anemia of the Rat. X. Hemoglobin Production and Iron and Copper Metabolism With Milk of Low Copper Content, *J. Biol. Chem.* 105: 343-354, 1934.
18. Mitchell, H., and Miller, L.: Studies in Nutritional Anemia. Quantitative Variations in Iron, Copper, and Manganese Supplements, *J. Biol. Chem.* 92: 421-434, 1931.
19. Hamre, C. J., and Miller, C. D.: The Spleen, Hemoglobin, and Erythrocytes in Nutritional Anemia of the Rat, *Am. J. Physiol.* 111: 578-589, 1935.
20. Hamre, C. J., and Miller, C. D.: Studies in Nutritional Anemia of the Rat. I. The Influence of the Spleen on Recovery, *Folia haematol.* 61: 224-238, 1938.
21. Smith, M. C., and Otis, L.: Effect of Adding Copper to the Exclusive Milk Diet Used in the Preparation of Anemic Rats Upon Their Subsequent Response to Iron, *J. Nutrition* 14: 365-371, 1937.
22. Orten, J. M., Underhill, F. A., and Lewis, R. C.: A Study of Certain Metals in the Prevention of Nutritional Anemia in the Rat, *J. Biol. Chem.* 96: 1-9, 1932.
23. Stein, H. B., and Lewis, R. C.: Is Erythropoietic Action of Copper Dependent Upon Adequate Supply of Iron in Diet? *Proc. Soc. Exper. Biol. & Med.* 29: 1174-1175, 1932.
24. Stein, H. B., and Lewis, R. C.: The Stimulating Action of Copper on Erythropoiesis, *J. Nutrition* 6: 465-472, 1933.
25. Stein, H. B., Radetsky, M. H., and Lewis, R. C.: Nutritional Anemia in Rats Alleviated by Evaporated Milk and Iron, *J. Dairy Science* 19: 117-124, 1936.
26. Von Haam, E., and Beard, H. H.: Pathological Changes in Liver and Spleen in Nutritional Anemia in Rat, *Proc. Soc. Exper. Biol. & Med.* 31: 639-643, 1934.
27. Fitz-Hugh, T., Jr., Robson, G. M., and Drabkin, D. L.: Hemoglobin Production. IV. Evaluation of Therapeutic Agents in Anemia, Due to Milk Diets, Based on the Study of the Blood and Bone Marrow of Rats From Birth to Maturity, *J. Biol. Chem.* 103: 617-628, 1933.
28. Elvehjem, C. A., and Kemmerer, A. R.: An Improved Technique for the Production of Nutritional Anemia in Rats, *J. Biol. Chem.* 93: 189-193, 1931.
29. Klieneberger, W. C.: *Die Blutmorphologie der Laboratoriumstiere*, Leipzig, 1927, Johann Ambrosius Barth.
30. Scarborough, R. A.: The Blood Picture of Normal Laboratory Animals; a compilation of published data, *Yale J. Biol. & Med.* 3: 63-80, 1930.
31. Ponder, E.: *The Mammalian Red Cell and the Properties of Haemolytic Systems*, vol. 6, Berlin, 1934, Verlagsbuchhandlung Gebrüder Borntraeger.
32. Krumbhaar, E. B.: Observations on the Nature of Post-operative Leucocytosis in the Dog, *Ann. Surg.* 66: 133-142, 1917.
33. Wintrobe, M. M.: *Clinical Hematology*, Philadelphia, 1946, Lea & Febiger.
34. Huggins, C., and Blockson, B. H., Jr.: Changes in Outlying Bone Marrow Accompanying a Local Increase of Temperature Within Physiological Limits, *J. Exper. Med.* 64: 253-274, 1936.
35. Doan, C. A., Cunningham, R. S., and Sabin, F. R.: Experimental Studies on the Origin and Maturation of Avian and Mammalian Red Blood Cells, Carnegie Institution of Washington, Contributions to Embryology 16: 163-226, 1925.
36. Jordan, H. E., and Johnson, E. P.: Erythrocyte Production in the Bone Marrow of the Pigeon, *Am. J. Anat.* 56: 71-95, 1935.

37. McDonald, J. G.: Avian Bone Marrow With Particular Reference to Red Cell Development, *Am. J. Anat.* 65: 291-307, 1939.
38. Kandel, E. V., and LeRoy, G. V.: Limitations of Biopsy of the Sternal Marrow, *Arch. Int. Med.* 64: 121-135, 1939.
39. Bock, H. E., and Malamos, B.: Beitrag zur Megaloblastenfrage, *Folia haemat.* 62: 408-429, 1939.
40. Sabin, F. R., and Miller, F. R.: Normal Bone Marrow, in Downey, Hal (editor): *Handbook of Hematology*, New York, 1938, Paul B. Hoeber, Inc., vol. 3, pp. 1790-1838.
41. Maximow, A.: Untersuchungen über Blut und Bindegewebe. I. Die frühesten Entwicklungsstadien der Blut- und Bindegewebszellen beim Säugetiere-embryo, bis zum Anfang der Blutbildung in der Leber, *Arch. f. mikr. Anat.* 73: 444-561, 1909.
42. Bloom, W., and Bartelmez, G. W.: Hematopoiesis in Young Human Embryos, *Am. J. Anat.* 67: 21-53, 1940.
43. Dantchakoff, W.: Untersuchungen über die Entwicklung des Blutes und Bindegewebe bei den Vögeln. I. Die erste Entstehung der Blutzellen beim Hühnerembryo und der Dottersack als Blutbildendes Organ, *Anat. Hefte* 37: 473-589, 1908.
44. Sabin, F. R.: Studies on the Origin of the Blood-Vessels and of Red Blood-Corpuscles as Seen in the Living Blastoderm of Chicks During the Second Day of Incubation, *Carnegie Institution of Washington, Contributions to Embryology* 9: 213-262, 1920.
45. Doan, C. A.: The Circulation of the Bone-Marrow, *Carnegie Institution of Washington, Contributions to Embryology* 14: 27-47, 1922.
46. Peabody, F. W.: A Study of Hyperplasia of the Bone Marrow of Man, *Am. J. Path.* 2: 487-501, 1926.
47. Bloom, W.: Embryogenesis of Mammalian Blood, in Downey, Hal (editor): *Handbook of Hematology*, New York, 1938, Paul B. Hoeber, Inc., vol. 2, pp. 865-922.
48. Maximow, A.: Untersuchungen über Blut und Bindegewebe. III. Die embryonale Histogenese des Knochenmarks der Säugetiere, *Arch. f. mikr. Anat.* 76: 1-113, 1910.
49. Drinker, C. K., Drinker, K. R., and Lund, C. C.: The Circulation of the Mammalian Bone-Marrow, *Am. J. Physiol.* 62: 1-92, 1922.
50. Bunting, C. H.: Experimental Anemias in the Rabbit, *J. Exper. Med.* 8: 625-646, 1906.
51. Key, J. A.: Studies on the Erythrocytes, With Special Reference to Reticulum, Polychromatophilia, and Mitochondria, *Arch. Int. Med.* 28: 511-549, 1921.
52. Downey, H.: The Myeloblast. Its Occurrence Under Normal and Pathological Conditions, and Its Relation to Lymphocytes and Other Blood Cells, in Downey, Hal (editor): *Handbook of Hematology*, New York, 1938, Paul B. Hoeber, Inc., vol. 3, pp. 1965-2041.
53. Cunningham, R. S., Sabin, F. R., and Doan, C. H.: The Development of Leucocytes, Lymphocytes, and Monocytes From a Specific Stem-Cell in Adult Tissues, *Carnegie Institution of Washington, Contributions to Embryology* 16: 227-276, 1925.
54. Sabin, F. R., Miller, F. R., Smithburn, K. C., Thomas, R. M., and Hummel, L. E.: Changes in the Bone Marrow and Blood Cells of Developing Rabbits, *J. Exper. Med.* 64: 97-120, 1926.
55. Downey, H.: The Occurrence and Significance of the "Myeloblast" Under Normal and Pathological Conditions, *Arch. Int. Med.* 33: 301-313, 1924.
56. Block, M.: An Experimental Analysis of Hematopoiesis in the Rat Yolk Sac, *Anat. Rec.* 96: 289-312, 1946.

# LYMPHOCTOGENESIS IN HUMAN LYMPH NODES

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THE origin, the development, and the widely varied morphologic changes, which are manifest during lymphocytemorphosis, have been and still are some of the most fundamental problems in all hematology. Classifications of the various types of cells grouped under the term lymphocyte are notoriously unsatisfactory, because lymphocytes lack the easily demonstrable specific characteristics such as the specific granules of granulocytes or the hemoglobin of developing erythrocytes. Although this lack of convincingly identifiable cytologic landmarks must be accepted as inevitable, the recent experiments showing that lymphocytes contain a protein identical with normal serum gamma globulin<sup>1, 2</sup> and that antibodies are present in the lymphocytes of immunized animals<sup>3, 4</sup> are dramatic examples of the importance of lymphocytes, examples which illustrate the need for renewed efforts at a clearer understanding of lymphocytemorphosis.

The origin of the lymphocyte in lymphatic tissue of laboratory animals and a comparison of the various types of lymphocytes to the myeloblasts of the bone marrow have been the subjects of research on the part of Downey and co-workers.<sup>5-12</sup> From these investigations and from studies of both blood and lymph nodes in infectious mononucleosis<sup>13, 14</sup> and in subacute lymphatic leukemia,<sup>15</sup> it was inferred that lymphocytemorphosis in nonleukemic human nodes probably closely simulated that which occurs in normal lymph nodes of rabbits and guinea pigs. The voluminous literature pertaining to the problems involved is partially reviewed in most of the publications but is most complete in the latest articles.<sup>11, 12</sup>

The present report is a summary of a study of lymphocytemorphosis in human lymph nodes. The various cell types involved were compared with the myeloblast of human bone marrow, with the immature and mature lymphocytes of the lymph nodes of rabbits and guinea pigs, and with the types of lymphoid cells seen in the lymph nodes, blood, and bone marrow of cases of infectious mononucleosis, chronic lymphatic leukemia, and acute and subacute lymphatic leukemias.

## MATERIALS AND METHODS

The 55 human lymph nodes studied were surgical specimens from which imprint preparations were made within a few minutes after their removal. They included 43 which showed no evidence of lymphoblastoma, 2 from chronic lymphatic leukemia, 9 from infectious mononucleosis (including the 8 nodes studied by Downey and Stasney<sup>14</sup>), and 1 from subacute lymphatic leukemia (that described by Stasney and Downey<sup>15</sup>). Eleven lymph nodes from rabbits and guinea pigs were useful in comparative studies; they have been described elsewhere.<sup>12</sup>

The 43 nonleukemic nodes included nodes from the lesser curvature of the stomach (4), the inguinal region (10), the axilla (24), and other locations (5). In all but four of the cases

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in which the nodes showed no evidence of leukemia, the patients' clinical and hematologic findings were investigated and found to be within the range of those expected with the varied causes of hospitalization. In no case was there any evidence of leukemia.

Direct smears of bone marrow and smears made of the myeloid-erythroid layer obtained from heparinized centrifuged bone marrow<sup>16</sup> from a wide variety of conditions other than leukemia were also examined in order that the various morphologic variations of the myeloblast might be correlated with the variations in the least differentiated types of lymphocytes visible in the nodes.

During the course of this investigation, blood and bone marrow received for diagnosis was also considered critically with reference to the various types of immature lymphoid cells encountered. The pathologic material reviewed which helped clarify the present problem included: (1) 50 cases of chronic lymphatic leukemia: blood, 34; blood and bone marrow, 16. (2) 31 cases of subacute or acute lymphatic leukemia: blood, 21; blood and bone marrow, 10. (3) 34 cases of infectious mononucleosis: blood, 33; blood and bone marrow, 1. (4) 12 cases of brucellosis: blood, 3; blood and bone marrow, 9.<sup>17</sup>

In order to determine the possible influence of blood on the cells from lymph nodes, a small amount of material from the mesenteric lymph node of a rabbit was scraped from a cut surface of the node and mixed with a small amount of blood. Smears were made from this mixture.

Lymph node imprints and smears of blood and bone marrow were stained with either the May-Grünwald-Giemsa combination or with Wright's stain. Lymph nodes were fixed in Helly's fluid, cut at 5 micra, and stained with hematoxylin and eosin or with Dominici's stain.

#### OBSERVATIONS

*Lymph Node Sections.*—Photomicrographs of sections of the lymph nodes have not been included because the development of lymphocytes from reticular cells has already been exhaustively studied by earlier investigators. The following works are singled out because they best illustrate the problem in question here. Downey and Weidenreich<sup>5</sup> described and included beautiful illustrations (their Figs. 2 and 3) of the transformation of fixed reticular cells to lymphocytes in sections of guinea pig lymph nodes. Thiel and Downey<sup>18</sup> described and illustrated the transformation of mesenchymal cells to hemocytoblasts and lymphocytes in the spleen of the pig embryo. Maximow<sup>19</sup> gave excellent illustrations of the types of cells seen in germ centers of rabbit (his Figs. 7 and 8) and human (his Figs. 9 and 10) lymph nodes and in a germ center of human tonsil (his Fig. 11). Maximow also showed the formation of large lymphocytes from the embryonic reticular syncytium in a newly formed lymph nodule in the mesentery of a rabbit (his Fig. 12). Klemperer<sup>20</sup> described and illustrated the transition from fixed reticular cells to hemocytoblasts in human pathologic lymph nodes and spleen.

*Histopathology of Nonleukemic Nodes.*—Since the development of lymphocytes rather than the types of pathologic changes occurring in lymph nodes is the subject of this report, the histopathologic changes observed in the lymph nodes can be considered only very briefly. None of the nodes examined was completely normal. Two nodes showed changes which might have resulted from mild physiologic (?) inflammation or age. Imprints from these nodes showed no evidence of active regeneration of lymphocytes. Five nodes which showed evidence of acute lymphadenitis were more normal in structure than any of the other specimens. Two of these nodes contained a fairly large number of small

germinal centers, and imprints showed an increase in the number of developing lymphocytes. Thirty-four nodes showed evidence of simple hyperplasia accompanied by chronic inflammatory changes. Of these thirty-four nodes, twenty-six were draining carcinomata, but only four of the latter showed the presence of tumor cells. The thirty-four simple hyperplastic nodes were those best adapted to this problem in that they all, regardless of the precipitating cause of hyperplasia, contained germinal centers in various stages of activity and showed increased numbers of developing lymphocytes in imprint preparations. Particularly interesting was the fact that the nodes draining the carcinomatous lesions showed pronounced lymphoid hyperplasia and many very large germinal centers, some of which were comparable in size to those seen in follicular lymphoblastoma. Only a very small number of macrophages containing the tingible Körper of Flemming<sup>21</sup> were seen in the large number of these centers which were studied. The centers were not reaction centers in the proper sense; they were actively engaged in the production of lymphocytes, for they were filled with reticular cells and transitional stages between reticular cells and lymphocytes, and mitoses were numerous. Because some of the imprints from these nodes contained large aggregates of lymphoid cells including primarily reticular cells, reticular lymphocytes, and cells in various stages of mitosis, it was concluded that actual *germinal centers had been imprinted*, and the comparison of the immature cells in sections and in imprints could be made with considerable assurance.

Nine nodes, biopsy specimens from cases of infectious mononucleosis, showed the histopathologic alterations ascribed to this disease,<sup>14</sup> and the imprints and smears showed a great increase in both the numbers of hematopoietic reticular cells and reticular lymphocytes<sup>22</sup> and of leukocytoid lymphocytes of various types.<sup>13</sup>

*Transformation of Reticular Cells to Lymphocytes.*—It is recognized that undifferentiated, primitive, fixed, or syncytial reticular cells are capable of transforming either to phagocytic cells or to hematopoietic cells. In sections of the lymph nodes, reticular cells, even while still in syncytial arrangement, could be seen to have undergone partial differentiation. The differentiation to phagocytic cells could be recognized by the presence of slight coarsening of the nuclear chromatin and increased vacuolization of the cytoplasm as well as by the presence of ingested particles. The differentiation to lymphocytes was manifested both by coarsening of nuclear chromatin and by increased basophilia of the cytoplasm. Although these changes could be seen in fixed or syncytially arranged reticular cells, more often the differentiating reticular cells were free. In imprint preparations, undifferentiated reticular cells which were apparently free could also be found (Fig. 1).

The transformation of undifferentiated reticular cells to lymphocytes as it occurs in human lymph nodes was found to be essentially similar to that previously described for the nodes of rabbits and guinea pigs.<sup>12</sup> The transformation was a gradual process involving many morphologic cell types which differed but slightly from one another. The stages in the transformation appeared to include (1) undifferentiated reticular cells, (2) hematopoietic reticular cells,

(3) reticular lymphocytes, and (4) lymphocytes identical with those of the blood. Mitoses were found to be most numerous in cells which, because of their size and the character of their cytoplasm (Fig. 11), are believed to be hematopoietic reticular cells and reticular lymphocytes rather than the common large lymphocytes of the blood. No cells morphologically identical with the myeloblast (Figs. 14, 14', 15) of the bone marrow or with the lymphoblast (Figs. 16, 16', 17) of acute and subacute lymphatic leukemias were found to participate in the regeneration of lymphocytes in the nonleukemic nodes.

The undifferentiated reticular cell (Fig. 1) is a large cell which possesses a relatively large amount of cytoplasm. Its nucleus, like those of the lymphoid cells derived from it, is generally round or oval in shape. The nuclear chromatin is fine in character, stains a light reddish violet, and is distinctly demarcated from the pale pink-staining and relatively abundant parachromatin (karyoplasm). The chromatin pattern is delicate. In areas, it appears to be net- or sievelike; however, generally small clumps, chains, and strands of chromatin are also present, and the resulting nuclear pattern is not uniform as is that of most myeloblasts. Nucleoli may or may not be present. They are variable in size but are usually small blue-staining bodies which are partially obscured by overlying chromatin. The nuclear membrane is fine but distinct.

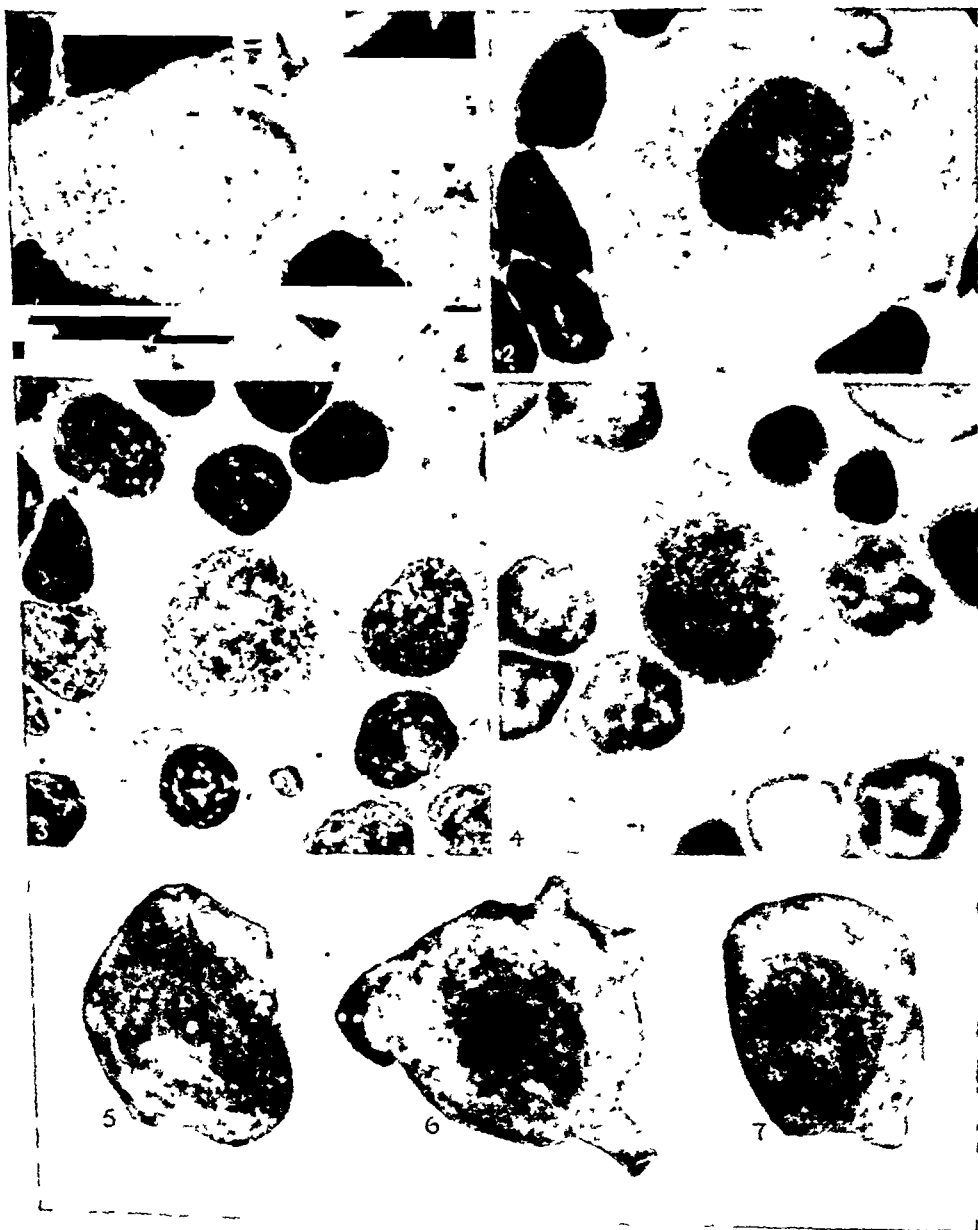
The cytoplasm of imprinted undifferentiated reticular cells is frequently damaged (Fig. 1); its actual boundaries are often not clearly visible. It appears to be watery, consisting of an abundant hyaloplasm with only a small amount of faintly basophilic spongioplasm. Vacuoles may be present; azure granules also can sometimes be found.

Figs. 2 and 2' show a cell very similar to the undifferentiated reticular cell. It has distinct cytoplasmic boundaries. Cells, not illustrated here, which occurred in the fields surrounding this cell contained phagocytosed material. Although the reticular cell in these figures might be considered an undifferentiated cell, in all probability it is a potential phagocyte (histiocyte). It is included in order best to illustrate the type of aggregation of chromatin which can be seen in the nuclei of reticular cells. (Compare the nuclear pattern of this cell with that of the hematopoietic reticular cell in Figs. 7 and 7'.)

In the transformation of the undifferentiated reticular cell to a one capable of forming blood cells, the most conspicuous change is a decrease in the amount of cytoplasm. The cytoplasm of hematopoietic reticular cells may be sparse (Figs. 3 and 4) but usually it is abundant (Figs. 5, 6, 7, 7'). Although basophilia of the cytoplasm is variable, most of the hematopoietic reticular cells have a grey-blue cytoplasm which is generally mottled or flaky in appearance and which often contains vacuoles (Figs. 3, 4, 5, 6, 7, 7'). The nuclear pattern may be almost identical with that of the undifferentiated reticular cell. (Compare nuclei of cells in Figs. 1 and 3.) The various cells classified as hematopoietic reticular cells because they resemble undifferentiated reticular cells and do not show indisputable evidence of transforming to lymphocytes are shown in Figs. 3, 4, 5, 6, 7, and 7'.

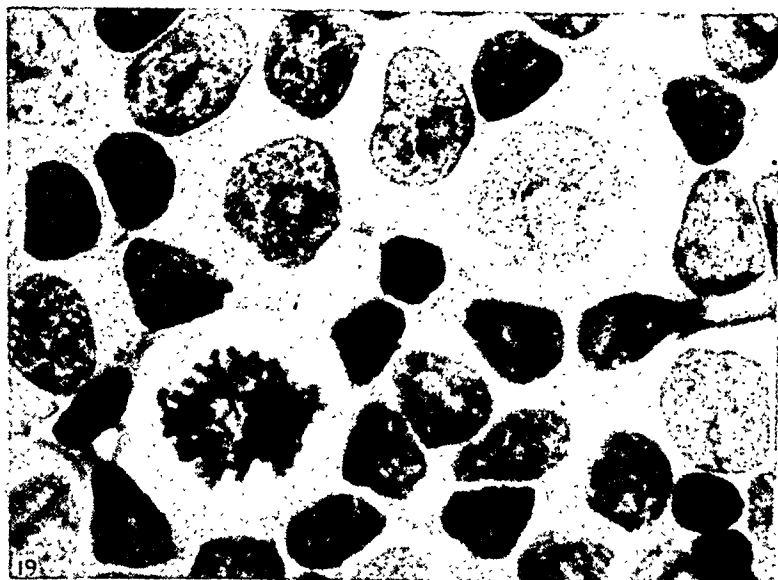
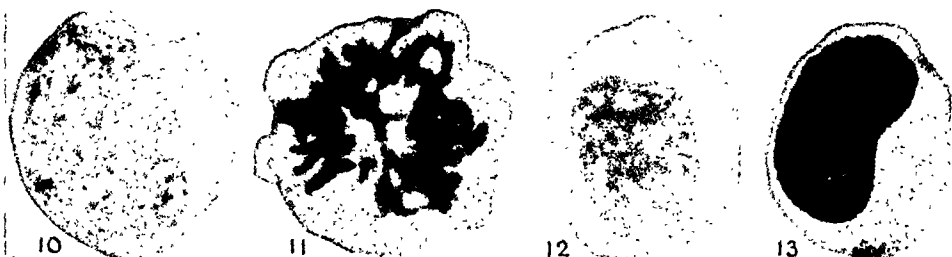
The next classifiable stage of differentiation is the reticular lymphocyte (Figs. 8 to 10). This cell has morphologic characteristics similar to those of

reticular cells and of lymphocytes. The recognition of this cell type as one distinct from the lymphoblasts of acute leukemia is of prime importance because small percentages of reticular lymphocytes may occur in the peripheral blood (Figs. 12, 12', 13) in benign lymphatic reactions.



Figs 1-7.—Cell types occurring in imprints of human nonleukemic lymph nodes which showed variable degrees of hyperplasia. All photomicrographs taken at same magnification ( $\times 1440$ ).

- 1.—Undifferentiated reticular cell.
- 2.—Histiocytic reticular cell.
- 3.—Hematopoietic reticular cell. Lymphocytes. Plasma cell.
- 4-7.—Hematopoietic reticular cells.



Figs. 8-10.—Reticular lymphocytes from imprints of hyperplastic human lymph nodes ( $\times 1440$ ).

Fig. 11.—Mitosis in hematopoietic reticular cell or reticular lymphocyte ( $\times 1440$ ). Imprint of hyperplastic human lymph node.

Figs. 12 and 13.—Reticular lymphocytes ( $\times 1440$ ) from blood in cases of infectious mononucleosis.

Figs. 14 and 15.—Myeloblasts ( $\times 1440$ ) from nonleukemic human bone marrow.

Figs. 16 and 17.—Lymphoblasts ( $\times 1440$ ) from blood in cases of acute lymphatic leukemia.

Fig. 18.—Lymphocyte ( $\times 1440$ ). Imprint of hyperplastic human lymph node.

Fig. 19.—Imprint of lymph node from chronic lymphatic leukemia. Hematopoietic reticular cells, reticular lymphocytes, lymphocytes, and mitosis ( $\times 1440$ ).

Narrow-bodied cells similar to the cell at the right in Fig. 8 were most similar to the myeloblasts of the marrow and to the lymphoblasts of acute and subacute lymphatic leukemias. These narrow-bodied immature lymphoid cells differ from lymphoblasts in that considerable aggregation of chromatin is present. However, cells of this type may be encountered in the peripheral blood in acute and subacute lymphatic leukemias. It is assumed that these cells represent immature lymphocytes, but their phase of maturity is such that it is not possible to determine whether they have been derived from reticular cells or from lymphoblasts.

The stages occurring during the transition of reticular lymphocytes to cells which exhibit the characteristic morphology of the normal lymphocytes of the blood are numerous. The changes include a diminution in the cell size and in the size of both nucleus and cytoplasm. The nuclear chromatin becomes coarse and is arranged in heavy clumps of variable size. The latter stain deeply and tend to blend with the relatively sparse parachromatin. The nuclear membrane is coarse and distinct; nucleoli are not visible. The cytoplasm is colorless or pale blue and more hyaline in appearance. (Note lymphocytes in Figs. 18 and 3.)

*Comparison to Myeloblasts.*—As has been stated, no isomorphs of the myeloblasts of human bone marrow were found in the imprints of human non-leukemic lymph nodes. There is, as is commonly recognized, a marked similarity

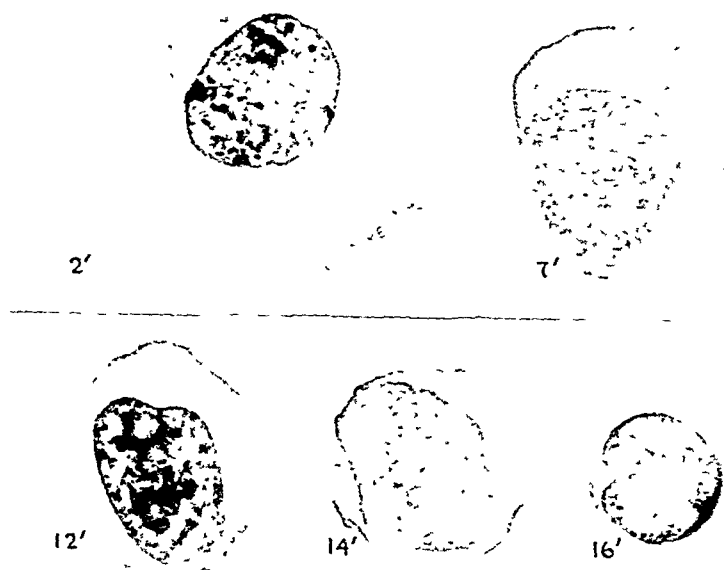


Fig. 2'.—Histiocytic reticular cell, same as Fig. 2.

Fig. 7'.—Hematopoietic reticular cell, same as Fig. 7.

Fig. 12'.—Reticular lymphocyte, same as Fig. 12.

Fig. 14'.—Myeloblast, same as Fig. 14.

Fig. 16'.—Lymphoblast, same as Fig. 16.

(Drawn from original cells with the aid of photomicrographs. Magnification,  $\times 1440$ .)

between blast forms of all types, and the morphologic characteristics which distinguish the hematopoietic reticular cell from the myeloblast are neither numerous nor prominent. Since both myeloblasts and lymphoblasts may be derived from reticular cells, morphologic evidence of the reticular derivation of either of these cells is sometimes present. The uniformity of nuclear structure in both myeloblasts and lymphoblasts might well be the result of repeated mitoses without subsequent differentiation.

Table I is included in order to facilitate comparison of the morphologic characteristics most commonly encountered in hematopoietic reticular cells, reticular lymphocytes, large lymphocytes, and myeloblasts or lymphoblasts. These characteristics are, of course, simply average findings.

TABLE I. COMPARISON OF IMMATURE LYMPHOCYTES WITH LARGE LYMPHOCYTE AND MYELOBLAST

Cell type	Hematopoietic reticular cell	Reticular lymphocyte	Large lymphocyte	Myeloblast or lymphoblast
Cell size	23 by 19 micra	20 by 17 micra	14 by 12 micra	16 by 15 micra
Nuclear size	17 by 14 micra	16 by 13 micra	11 by 9 micra	14 by 13 micra
Nuclear shape	Round or oval	Round or oval	Round or oval	May be irregular
Nuclear pattern	Irregular	Irregular	Irregular	Uniform
Chromatin				
Staining	Light reddish violet	Reddish violet	Purple	Light reddish violet
Nature	Small particles of variable size; chains; strands of granules	Similar to hematopoietic reticular cell but larger masses; more strands of granules	Large angular masses; only very few chains	Fine, sieve- or netlike; sometimes granular or stippled
Parachromatin				
Amount and nature	Abundant and very distinct	Abundant and distinct	Sparse and indistinct	Not overly abundant but distinct
Staining	Pale pink	Pale pink to pale reddish violet	Pale reddish violet to gray blue	Pale pink
Nucleoli				
Number	Variable	Variable	Not visible	Variable
Diameter	1 to 7 micra	1 to 7 micra		1 to 3 micra
Staining	Pale blue	Pale blue to blue		Pale blue
Shape	Rounded or irregular	Rounded or irregular		Round or oval
Character	Indistinct	Indistinct		Distinct
Nuclear membrane	Fine, distinct	Coarse, less distinct	Coarser, less distinct	Very fine, indistinct
Cytoplasm				
Staining	Gray blue to colorless	Deep blue to colorless	Pale blue to colorless	Pale blue
Character	Mottled and flaky	Mottled and flaky	Usually homogeneous	Usually homogeneous
Vacuoles	Common	Common	Uncommon	Uncommon
Azure granules	Rare	Rare	Uncommon	Common in myeloblast; uncommon in lymphoblast

*Lymphocytogenesis in Animal Lymph Nodes.*—Lymphocytogenesis in human lymph nodes was found to be remarkably similar to lymphocytogenesis in the nodes of rabbits and guinea pigs. In the hyperplastic but nonleukemic human nodes, hematopoietic reticular cells were, however, more numerous than

in the nodes of rabbits and guinea pigs. In the nodes of rabbits and guinea pigs, reticular lymphocytes, most of which possessed a more deeply basophilic cytoplasm than did the reticular lymphocytes of human nodes, were far more numerous than hematopoietic reticular cells, and mitoses were most numerous in these reticular lymphocytes. No important morphologic changes were produced by mixing the cells from a rabbit's lymph node with blood.

*Lymphocytogenesis in Infectious Mononucleosis.*—Lymphocytogenesis in infectious mononucleosis resembled that seen in both the simple hyperplastic human nodes and the animal nodes. As would be expected, however, there was more evidence of active regeneration of lymphocytes in the nodes from infectious mononucleosis than in any of the other nonleukemic nodes. The following features were recognized: (1) Hematopoietic reticular cells and reticular lymphocytes were more numerous in many of the lymph nodes from infectious mononucleosis than in most of the other nonleukemic nodes, and reticular lymphocytes were far more numerous than hematopoietic reticular cells. (2) The cytoplasm of the immature cells seen in infectious mononucleosis generally showed pronounced basophilia, and in many of these cells the cytoplasm was almost completely homogeneous. (3) Some of the extremely basophilic reticular lymphocytes appeared to function as precursors of plasma cells. However, transitions between these basophilic reticular lymphocytes (plasmablasts of Moeschlin<sup>23, 24</sup>) and the Type I leukocytoïd lymphocyte of Downey and McKinlay<sup>12</sup> were also prominent. (4) Mitoses were more numerous in many of the lymph nodes from infectious mononucleosis than in any of the other nonleukemic nodes. Many more mitoses appeared to occur in the reticular lymphocytes of the lymph nodes of infectious mononucleosis than in the reticular lymphocytes of the other nonleukemic nodes. (5) In the lymph nodes from infectious mononucleosis, transitions between the reticular lymphocytes and the leukocytoïd lymphocytes of infectious mononucleosis were numerous, whereas such transitions were very rare in the other nonleukemic nodes.

The bone marrow from one case of infectious mononucleosis showed a relative (29.5 per cent) and absolute lymphocytosis (myeloid-erythroid volume, 18.5 per cent). Hematopoietic reticular cells, reticular lymphocytes, and leukocytoïd lymphocytes were numerous. All three of these cell types were morphologically distinct from the myeloblasts of this marrow.

*Lymphocytogenesis in Chronic Lymphatic Leukemia.*—Lymph nodes from two cases which exhibited the typical clinical and hematologic findings of chronic lymphatic leukemia were studied. The node from the first case had been palpable for a long period of time; the node from the second case had become noticeable in a period of about three weeks. The former node was considered to be relatively static, and it will be designated Node S; the latter was one which had undergone rapid growth, and it will be designated Node G. Each of these nodes showed in section preparations the characteristic features of chronic lymphatic leukemia. In Node G, however, reticular hyperplasia and mitoses were more prominent.

The imprint material from Nodes S and G showed such a striking lack of similarity that the relatively similar appearing sections from these two nodes seemed almost incomprehensible.



Node S showed an almost completely monotonous picture of large lymphocytes. These lymphocytes had a nuclear pattern only slightly different from that of the large lymphocytes of normal blood. The chromatin masses were neither as heavy nor as deeply stained as those of ordinary lymphocytes, and the parachromatin was more clearly visible than is that of normal lymphocytes. The cytoplasm was less abundant than that of most lymphocytes, but it was not as sparse as that of the lymphoblasts of acute lymphatic leukemia or as that of many of the myeloblasts of human nonleukemic bone marrow. The most prominent feature was not, however, this slightly abnormal appearance of the lymphocytes, for lymphocytes of this type can be found in almost any lymph node imprint. The striking feature was the lack of variation in cell type. Cells which appeared less differentiated than these large lymphocytes were present, but they were neither particularly numerous nor conspicuous. Some resembled the hematopoietic reticular cells and reticular lymphocytes of nonleukemic nodes, but most of them showed an even more striking clarity of nuclear detail than that seen in similar cells from nonleukemic nodes. Nucleoli, particularly, seemed more distinct. Yet, none of the cells seen was identical with the myeloblast (Figs. 14, 14' 15) of the bone marrow or with the lymphoblast (Figs. 16, 16', 17) of acute lymphatic leukemia. All of the immature cells showed morphologic patterns interpreted as being indicative of their close relationship to reticular cells.

Node G, in contrast, showed a picture which was completely lacking in the monotonous uniformity of cell type which was seen in Node S. Although all sizes of lymphocytes were present, the lymphocytic picture was overshadowed by tremendous numbers of cells many of which were almost identical to the hematopoietic reticular cells and reticular lymphocytes that occurred in the nonleukemic nodes. (Note, for example, the striking similarity of the largest cell shown in Fig. 19 to the hematopoietic reticular cell shown in Figs. 7 and 7'. Compare also the large cells in Figs. 5 and 19; both have large and irregularly shaped, indistinct nucleoli, and both have vacuoles in both nuclei and cytoplasm. Finally, compare the mitotic figure in Fig. 19 with that in Fig. 11.) Many cells which were morphologically identical with the hematopoietic reticular cell in Figs. 7 and 7' were present in Node G. Everywhere throughout all of the imprints of Node G hematopoietic reticular cells, along with intermediate stages between them and lymphocytes, and mitoses were numerous. In fact, one might compare the picture as a whole to one which could be produced by imprinting one mammoth germinal center. This degree of hyperplasia was not, however, encountered in any of the nonleukemic human or animal nodes. In imprints from Node G, as in those from Node S, there was a striking clarity of cytologic detail. Because it was suspected that this might be caused by some variation in technique, nonleukemic and leukemic nodes were stained simultaneously and in an identical manner. The resulting preparations showed that the method of staining was not the explanation for this phenomenon.

The degree of reticular hyperplasia which was evident in Node G is considered unusual for chronic lymphatic leukemia. Most of the nodes from chronic lymphatic leukemia which have been described and which are encountered in routine autopsy material show the monotonous uniformity of pattern seen in

Node S of this report. In the case from which Node G was removed, neither the patient's clinical condition nor his hematologic findings suggested an exacerbation of the leukemic process. The best explanation for the active regeneration of lymphocytes seen in the node seemed to be that the node had undergone recent rapid growth.

*Lymphocytogenesis in Acute and Subacute Lymphatic Leukemias.*—Lymphoblasts from acute lymphatic leukemias in which the peripheral blood smears showed large numbers of cells of this type are shown in Figs. 16, 16', and 17. These cells are very similar to the cells seen in the imprints of the biopsied lymph nodes studied by Stasney and Downey,<sup>15</sup> and they are also similar to many of the cells seen in the peripheral blood of their case. Since no lymph nodes from acute or subacute lymphatic leukemia were obtained during the course of this study, conclusions were based upon a comparison of the cell types seen in nonleukemic nodes with the lymphoblasts seen in the peripheral blood and in the bone marrow of cases of acute and subacute lymphatic leukemia and with the lymphoblasts seen in the imprints of the nodes studied by Stasney and Downey.

The nonleukemic nodes did not contain cells morphologically identical with the leukemic lymphoblasts. The leukemic nodes and the peripheral blood smears and bone marrow smears from the various cases of acute and subacute lymphatic leukemias contained large numbers of lymphoblasts many of which could not be distinguished from myeloblasts. In addition, the smears from the acute and subacute leukemias also contained occasional hematopoietic reticular cells and reticular lymphocytes as well as immature lymphocytes with narrow cytoplasmic bodies similar to the cell at the right in Fig. 8. The latter type of cell can be found in relatively normal and in hyperplastic nodes, in the blood, nodes, and marrow of lymphatic leukemia, and also occasionally in the blood of normal children.

#### REVIEW OF LITERATURE AND DISCUSSION

Publications presenting detailed morphologic studies of lymphocytogenesis as seen in both imprint and section preparations of surgically biopsied, human nonleukemic nodes have not been encountered. However, various investigators who have aspirated material from lymph nodes for diagnostic purposes have discussed regeneration of lymphocytes in normal nodes.

Fleischhacker and Klima<sup>25</sup> stated that "In order to get a criterion about the morphology of normal lymph nodes, we punctured patients with good available but otherwise unchanged lymph nodes." They found that smears of these normal nodes showed predominantly ripe lymphocytes, but a few larger, less mature forms were present and were interpreted as lymphoblasts. They made a distinction between myeloblasts of myelogenous leukemia and lymphoblasts, but they made no distinction between the lymphoblasts of normal nodes and those of lymphatic leukemia.

Tischendorf<sup>26</sup> confirmed some of the conclusions of the present study by stating that the puncture of healthy nodes was hardly possible and by adding that when lymph nodes are large enough to be punctured, they are probably pathologic. Tischendorf, however, also described typical lymphoblasts in nodes

and claimed they were increased in number in hyperplasias and in leukemias. He clarified his interpretation of the term lymphoblast by stating that in some leukemias, large cells with spongy nuclei containing intensively blue and distinct nucleoli, and whose protoplasm was delicate and often "fringed out," could be seen. These cells *may*, according to him, be stem cells which may be derived from the capillary endothelium. Of particular interest was the fact that Tischendorf also discussed a case of chronic lymphatic leukemia from which the lymph node material showed large numbers of immature cells (his stem cells and lymphoblasts). In lymph aspirated from the nodes of infectious mononucleosis, he found cells two times the size of lymphocytes with fine nuclei which "lie in a deep dark blue cytoplasm." These cells were regarded as characteristic cells of infectious mononucleosis which showed no resemblance to the immature lymphoid cells of relatively normal nodes. Tischendorf, like Fleischhacker and Klima,<sup>25</sup> considered myeloblasts and lymphoblasts as morphologically distinct cell types.

It is difficult to determine how much agreement there is between the present findings and those of the German authors.<sup>25, 26</sup> In the present study, the term lymphoblast is reserved, as it has been in the preliminary studies of lymphocytogenesis, for the immature lymphoid cell of acute and subacute lymphatic leukemias. The lymphoblast is considered to be a cell which is frequently morphologically indistinguishable from the myeloblast. Fleischhacker and Klima<sup>25</sup> and Tischendorf<sup>26</sup> claimed separate identity for myeloblasts and lymphoblasts, but they found lymphoblasts in normal as well as in leukemic nodes. Although Tischendorf claimed that the immature cells of infectious mononucleosis bore no resemblance to the immature cells of relatively normal nodes, it seems extremely likely that the cells which he observed in infectious mononucleosis would correspond to the hematopoietic reticular cells and reticular lymphocytes of the present report. As has been previously discussed, the immature lymphoid cells of infectious mononucleosis are, except for more intense cytoplasmic basophilia, almost identical to those seen in other hyperplastic nodes.

Moeschlin,<sup>24</sup> in his report on a study of three cases of infectious mononucleosis in which he studied aspiration material from lymph nodes, bone marrow, and spleen as well as the blood, showed that much of his work confirmed the previous work of Downey and Stasney.<sup>14</sup> However, in this paper and in a previous work on lymph node aspiration material from rubeola,<sup>23</sup> Moeschlin described two immature cells which he felt were specific cell types. The first of these cells was the plasmablast, and since he could see transitions between this cell and plasma cells, he felt that it was much more likely that the plasma cells formed in lymphatic tissue were derived from this plasmablast than from lymphocytes. As nearly as can be determined from his descriptions and his Fig. 4, *a*,<sup>23</sup> Moeschlin's plasmablast appears to correspond to some of the unusually basophilic hematopoietic reticular cells and reticular lymphocytes seen in the present investigation.

In view of Moeschlin's hypothesis, however, nodes of all types were studied in an effort to determine whether the large reticular elements should be considered as plasmablasts. Transitions between reticular lymphocytes and plasma cells were encountered, but transitions between lymphocytes and plasma cells

were also often seen in nodes where no cells resembling the plasmablasts of Moeschlin could be found, and cells corresponding well with his plasmablast were found in nodes which contained few or no plasma cells. Therefore, in spite of Moeschlin's excellent figures, it does not seem correct that he designate the extremely immature hematopoietic reticular cell as a specific plasmablast and ignore the fact that this cell may function as a parent cell for all the lymphatic elements of the nodes, including plasma cells. (Note plasma cell in Fig. 3.)

The second immature cell type was Moeschlin's<sup>24</sup> lymphatic monoblast. This cell (his Fig. 5, 1), which occurred in increased numbers in infectious mononucleosis appears to correspond to the reticular lymphocytes of the present investigation. Moeschlin called it a lymphatic monoblast because transitional stages occurred between it and a lymphatic cell (which showed an indented nucleus and coarser chromatin), the lymphatic monocyte. The latter cell corresponds to Downey and McKinlay's<sup>13</sup> leukocytoid lymphocyte, Type I. Here, again, terminology is confusing. Moeschlin's term lymphatic monoblast is an acceptable descriptive term, for certainly some of the leukocytoid lymphocytes of infectious mononucleosis resemble monocytes and might be called lymphatic monocytes. However, cells entirely similar to Moeschlin's lymphatic monoblasts (reticular lymphocytes of the present investigation) were numerous in nodes which were hyperplastic but which showed almost no lymphocytes with indented nuclei resembling the lymphatic monocytes of Moeschlin.

Although Moeschlin agreed with Downey and Stasney<sup>14</sup> that the majority of young cells in the nodes of infectious mononucleosis should not be called lymphoblasts, he found lymphoblasts in normal and in other nonleukemic nodes. It is difficult to determine just what type of cell his lymphoblast (Fig. 7, d<sup>23</sup>) actually is. His figure showed a cell approximately 17 micra in diameter with a narrow rim of cytoplasm and prominent nucleoli, but with relatively coarse chromatin. Although no cells identical with Moeschlin's lymphoblast were seen in the nonleukemic nodes studied, cells of about the same size, with relatively coarse chromatin and with nucleoli, were occasionally seen in the poorly preserved parts of the imprints. In these same areas, it was often possible to see nucleoli in lymphocytes of all sizes. Because the morphologic picture seemed to be one due to damage and distortion, cells of this type were not included in the present study.

Although literature dealing with lymphocyto-genesis in human nonleukemic nodes is sparse, there is a voluminous literature (the product largely of studies of animal or leukemic material) in which numerous authors express their views of the fundamentals of hematopoiesis. Downey discussed the early literature in 1927<sup>6</sup> and in 1938.<sup>11</sup> In a more recent publication,<sup>12</sup> it was again assumed that lymphocyto-genesis in human nonleukemic nodes might closely parallel that seen in the nodes of rabbits and guinea pigs, and the discussion included in that report considered the various theories proposed for regeneration of lymphocytes. Since the results of the present investigation support the conclusions drawn in that study, no similar discussion will be included here.

A few points, however, require further clarification. The introduction of new terms into a science already encumbered by terminology is not desirable. All precursors of lymphocytes obviously are lymphoblastic in function, but un-

fortunately the term lymphoblast has been used in the past and is used in present-day clinical hematology to identify the most prominent lymphoid cell found in the blood in acute and subacute lymphatic leukemias. Most hematologists recognize the marked similarity of lymphoblasts and myeloblasts. Since cells conforming in morphology to the common conception of the term lymphoblast were not found in the nonleukemic nodes, it seems unwise to employ the name lymphoblast in *any* discussion of normal lymphocytopoiesis. In an earlier publication,<sup>12</sup> lymphocyte regeneration was reconstructed as proceeding from undifferentiated reticular cells to reticular lymphocytes either directly or through a blast stage called the hematopoietic reticular cell or hemohistioblast. Mitoses were found to be most numerous in cells which appeared to be either hematopoietic reticular cells or reticular lymphocytes. Numerous transitional stages occurred between these two cell types, and the transition to ordinary lymphocytes was also a gradual one.

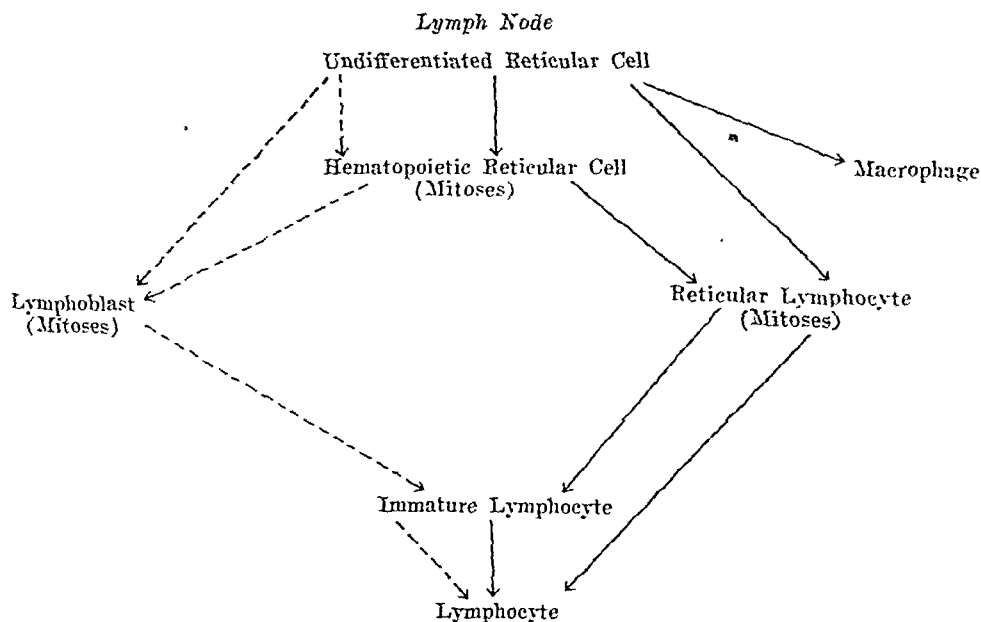
The problem is, for the most part, one of academic importance. However, in many instances an understanding of lymphocytopoiesis is extremely valuable from a diagnostic standpoint. Various conditions are associated not only with peripheral lymphocytosis but also with the presence of a small percentage of immature lymphoid cells in the peripheral blood. These conditions are considered lymphatic reactions, reactions in which occasional normal immature lymphoid cells, reticular lymphocytes, enter the circulation. Reticular lymphocytes have been encountered in infectious mononucleosis, brucellosis, infectious hepatitis, some acquired hemolytic anemias, and in conditions in which there was pronounced eosinophilia. Because reticular lymphocytes may contain nucleoli and may exhibit a fine nuclear pattern (Figs. 12, 12', 13), they are sometimes mistaken for lymphoblasts, and erroneous diagnoses of leukemia are made. When it is understood that these reticular lymphocytes are merely immature lymphoid cells which may occur in any hyperplastic lymph node and which, when in the peripheral circulation, are usually simply the result of a benign left shift in lymphocytopoiesis, many of the problems of diagnosis will be simplified.

One noteworthy exception exists. In most chronic lymphatic leukemias, immature lymphocytes were not numerous in the peripheral blood. When immature cells were present, they were more similar to the reticular lymphocytes of infectious mononucleosis and of human nonleukemic nodes than to the lymphoblasts of acute leukemias. In fact, as has been stressed elsewhere,<sup>22</sup> there are cases of chronic lymphatic leukemia in which the peripheral blood so closely resembles that of some cases of infectious mononucleosis that a differential diagnosis on the basis of an examination of the blood smear is not always possible. The findings of reticular hyperplasia in node G is, therefore, not surprising; it serves merely to clarify previous impressions. Data available at the present time suggest that in chronic lymphatic leukemia, lymphocytopoiesis closely parallels that seen in nonleukemic nodes. The clarity of morphologic detail in both mature and immature cells is, however, striking in many cases. It is possible that this clarity is the result of frequent mitoses with the production of enormous numbers of almost identical cells.

The presence of lymphoblasts in acute and subacute lymphatic leukemia and their absence in nonleukemic nodes is difficult to explain. Since they were not found in the present investigation, it is assumed that they do not occur in significant numbers in nonleukemic nodes. Transitional stages between the reticular lymphocytes of nonleukemic nodes and normal lymphocytes are often morphologically identical with transitional stages between lymphoblasts and normal appearing lymphocytes. Only the immature cells differ. That reticular cells may differentiate to lymphoblasts which undergo numerous mitoses and little if any differentiation is one explanation of the phenomenon.<sup>15</sup> It appears to be a very likely explanation, for in nodes, marrow, or blood from subacute and acute lymphatic leukemias, a few cells which show evidence of reticular origin can almost always be found.

Transitional stages encountered in this study can be organized into a diagrammatic type of working hypothesis (Table II).

TABLE II. LYMPHOCYTOGENESIS



——, Transitional stages encountered in simple hyperplastic nodes, in nodes from infectious mononucleosis, and in nodes from chronic lymphatic leukemia.

- - - - -, Transitional stages encountered in acute and subacute lymphatic leukemias.

## SUMMARY

Relatively normal human lymph nodes, like the normal lymph nodes of rabbits and guinea pigs, do not contain cells morphologically identical with the myeloblasts of the bone marrow. The immature lymphoid cells (hematopoietic reticular cells and reticular lymphocytes) which are the progenitors of lymphocytes are morphologically distinguishable from myeloblasts. On this basis, it can be said that morphologic and functional dualism exists in normal adult

hematopoiesis; the hematopoietic reticular cell functions as a stem cell for lymphocytes, and the myeloblast functions as the stem cell for myeloid elements.

The results of the present investigation indicate that this condition prevails except in some cases of acute and subacute lymphatic leukemias where cells called lymphoblasts, many of which are morphologically indistinguishable from myeloblasts, occur in the blood, the lymph nodes, and the bone marrow.

#### REFERENCES

1. White, A., and Dougherty, T. F.: Influence of Hormones on Serum Proteins, Abstracts of Proc. Meet. Am. Chem. Soc., New York, September, 1944.
2. White, A., and Dougherty, T. F.: The Pituitary Adrenotrophic Hormone Control of the Rate of Release of Serum Globulins From Lymphoid Tissue, *Endocrinology* 36: 207, 1945.
3. Dougherty, T. F., Chase, J. H., and White, A.: The Demonstration of Antibodies in Lymphocytes, *Proc. Soc. Exper. Biol. & Med.* 57: 295, 1944.
4. Dougherty, T. F., and White, A.: Functional Alterations in Lymphoid Tissue Induced by Adrenal Cortical Secretion, *Am. J. Anat.* 77: (1): 81, 1945.
5. Downey, H., and Weidenreich, F.: Ueber die Bildung der Lymphocyten in Lymphdrüsen und Milz, *Arch. f. mikr. Anat.* 80: 306, 1912.
6. Downey, H.: On the Development of Lymphocytes in Lymph Nodes and Spleen, *Tr. Minn. Path. Soc.* 1: 91, 1912.
7. Downey, H.: The Occurrence and Significance of the "Myeloblast" Under Normal and Pathologic Conditions, *Arch. Int. Med.* 33: 301, 1924.
8. Downey, H.: The Myeloblast, Its Occurrence Under Normal and Pathological Conditions, and Its Relations to Lymphocytes and Other Blood Cells, *Folia haemat.* 34: 65, 145, 1927.
9. Downey, H.: The Myeloblast, in Cowdry, E. V., editor: *Special Cytology*, ed. 2, New York, 1932, Paul B. Hoeber, Inc., vol. 2, p. 651.
10. Downey, H.: The Regeneration of Lymphocytes, *Anat. Rec. (Suppl.)* 61: 14, 1935.
11. Downey, H.: The Myeloblast. Its Occurrence Under Normal and Pathological Conditions, and Its Relations to Lymphocytes and Other Blood Cells, in Downey, H., editor: *Handbook of Hematology*, New York, 1938, Paul B. Hoeber, Inc., vol. 3, p. 1963.
12. Sundberg, R. D., and Downey, H.: Comparison of Lymphoid Cells of Bone Marrow and Lymph Nodes of Rabbits and Guinea Pigs, *Am. J. Anat.* 70 (3): 455, 1942.
13. Downey, H., and McKinlay, C. A.: Acute Lymphadenosis Compared With Acute Lymphatic Leukemia, *Arch. Int. Med.* 32: 82, 1923.
14. Downey, H., and Stasney, J.: The Pathology of Lymph Nodes in Infectious Mononucleosis, *Folia haemat.* 54: 417, 1936.
15. Stasney, J., and Downey, H.: Subacute Lymphatic Leukemia, *Am. J. Path.* 11: 113, 1935.
16. Sundberg, R. D.: Sternal Aspiration, *Staff Meet. Bull., Hosp. Univ. Minnesota* 17 (26): 389, 1946.
17. Sundberg, R. D., and Spink, W. W.: The Histopathology of Lesions in the Bone Marrow of Patients Having Active Brucellosis, *Blood*. In press.
18. Thiel, G. A., and Downey, H.: The Development of the Mammalian Spleen, With Special Reference to Its Hemopoietic Activity, *Am. J. Anat.* 28: 279, 1921.
19. Maximow, A.: The Lymphocytes and Plasma Cells, in Cowdry, E. V., editor: *Special Cytology*, ed. 2, New York, 1932, Paul B. Hoeber, Inc., vol. 2, p. 601.
20. Klempner, P.: The Relationship of the Reticulum to Diseases of the Hematopoietic System, *Libman Anniv. Vols.* 2: 655, 1932.
21. Flemming, W.: Studien über Regeneration der Gewebe. I. Die Zellvermehrung in den Lymphdrüsen und verwandten Organen und ihr Einfluss auf deren Bau, *Arch. f. mikr. Anat.* 24: 50, 1885.
22. Sundberg, R. D.: Infectious Mononucleosis, *Detroit M. News, Educ. Issue* 32: 29, 1941.
23. Moeschlin, S.: Untersuchungen über Genese und Funktion der Blutplasmazellen anhand von Lymphdrüsen- und Sternalpunktaten bei Rubeolen, *Helvet. med. acta* 7: 227, 1940.
24. Moeschlin, S.: Die Genese der Drüsenfieberzellen (Mononucleosis infectiosa) an Hand von Drüsen-, Sternal-, und Milzpunktaten, *Deutsches Arch. f. klin. Med.* 187: 249, 1941.
25. Fleischhacker, H., and Klima, R.: Zellbilder von Lymphknotenpunktaten und ihre diagnostische Verwertbarkeit, *München. med. Wehnschr.* 84: 661, 1937.
26. Tischendorf, W.: Ueber die Verwertbarkeit von Lymphknotenpunktaten zur Differentialdiagnose der Lymphdrüsenkrankungen, *Deutsches Arch. f. klin. Med.* 183: 448, 1938.

# THE CLINICAL SIGNIFICANCE OF CELLULAR GIGANTISM IN HUMAN ERYTHROPOIESIS

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PLURINUCLEAR erythroblasts and pluripolar mitoses among erythroblasts in megaloblastic erythropoiesis have been described by a number of authors.<sup>1, 7, 12-16, 19, 20</sup> On the other hand, their occurrence in nonmegaloblastic erythropoiesis, which has been mentioned or illustrated by a few authors,<sup>13-15, 17, 18, 20</sup> is considered to be very unusual.<sup>5, 8, 11, 15</sup> In 1943 Limarzi and Levinson<sup>15</sup> reported a case in which they found what was considered to be a hitherto undescribed type of erythropoiesis in human sternal marrow. This was characterized by the formation of giant erythroblasts, giant plurinuclear erythroblasts having two or more nuclei, giant erythroblasts with lobulated nuclei, and abnormal mitoses with three or more poles. The erythroblasts appeared to be neither megaloblastic nor normoblastic; they were so abnormal and large that they were called gigantoblasts. The authors regarded the changes as irreversible and indicative of a cancerous process and proposed the terms erythroma or erythroblastoma. In 1944 Schleicher<sup>19</sup> reported two cases with similar atypical erythroblasts, chiefly of orthochromic type. He regarded them an expression of defunct reticulum, thereby implying that the changes were terminal or irreversible. Recently, Schwarz<sup>20</sup> reported a study of the question of cellular gigantism in human hematopoiesis based on observation of sternal marrow smears; he summarized the occurrence of giant plurinuclear erythroblasts and pluripolar mitoses among erythroblasts as phenomena independent of the type and condition of hematopoiesis. He concluded that gigantism does not depend on a morbid condition of erythropoiesis since it occurs in normal as well as in pathologic marrows. A somewhat contrary view regarding the significance of plurinuclear erythroblasts is expressed by Leitner<sup>13</sup> who states that atypical cell divisions are present mostly in serious disorders and are found in large numbers only in severe hemopathies.

In this laboratory we have observed a fairly regular appearance of plurinuclear erythroblasts in both normal and pathologic marrows, exclusive of the megaloblastic marrows in which it is generally agreed that these changes are common. In view of the alleged rarity of these conditions in nonmegaloblastic marrows (Limarzi and Levinson<sup>15</sup>) in contrast to its commonly observed occurrence in our experience and that of others (Schwarz<sup>20</sup> and Leitner<sup>13</sup>), and the divergence of opinion regarding their proper interpretation, it is desirable that material be reviewed for the purposes of shedding light on the following: (1) the incidence of the phenomena under question in normal and pathologic marrows, (2) the question of the reversibility of the changes, (3) the question of

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whether or not the changes are of agonal or terminal nature, (4) the question of whether or not erythropoiesis of the peculiar type here considered represents a new, different, or cancerous form of development, and (5) the presentation of data which may aid in the eventual interpretation of the clinical significance of such findings. For these objectives it is essential that cases for study are selected because of a significantly high incidence of plurinuclear erythroblasts, that more than a single marrow examination be available during life, and that none of the patients be in a terminal or agonal state. These criteria are fulfilled by the eight cases with pathologic marrows to be described in detail. In addition, control studies were carried out on eight normal individuals.

#### MATERIAL AND METHODS

The eight pathologic cases selected included one patient each with pernicious anemia, anemia associated with chronic blood loss, lymphoblastoma, monocytic leucemia, congenital hemolytic anemia before and after splenectomy, idiopathic thrombocytopenic purpura before and after splenectomy, cirrhosis of the liver, and acute hepatitis. The eight normal individuals selected for the control studies were healthy white males whose ages ranged from 18 to 53 years. They were chosen on the basis of their having had no serious illnesses and of having a sense of well-being, normal hematologic, chemical, and metabolic findings including the following: erythrocyte, leucocyte, platelet and reticulocyte counts, differential counts of leucocytes, bleeding and clotting times, capillary fragility, prothrombin and blood urea nitrogen levels, liver function tests, urinalyses, sedimentation rate, basal metabolic rate, electrocardiogram, stereoscopic chest x-ray, and thorough physical examination. In each case the sternal marrow was obtained, prepared, and examined by the methods previously described as routine for this laboratory.<sup>2</sup> Differential counts of the nucleated cells in the smears were based on examination of a minimum of 1,000 cells.

The age range of the control cases is not identical with that of the eight pathologic cases studied, but it is felt that they form a homogeneous age group with regard to sternal marrow findings, since Jacobsen's<sup>19</sup> study of bone marrows in seventy normal individuals ranging in age from 15 to 93 years reveals no significant variation due to differences of age. Furthermore, the fact that all the normal individuals in the control series are males does not detract from its reliability, since Segerdahl's<sup>21</sup> study of normal myelograms of 61 adult males and 49 adult females of comparable ages revealed only very slight variations in the myelograms due to difference of sex.

#### TERMINOLOGY

It has been pointed out that the phenomena of plurinuclearity and pluripolarity of mitosis are seen in both leucocytes and erythroblasts\* of the sternal marrow.<sup>13, 20</sup> The present study is confined to the interpretation of these changes affecting erythroblasts. In the papers already cited, various terms have been used to denote the abnormal erythroblasts. Limarzi and Levinson refer to a very large uninuclear precursor of the abnormal cells and the large plurinuclear erythroblasts as giant erythroblasts and giantoblasts after Ehrlich and Lazarus.<sup>6</sup> Schleicher refers to the large plurinuclear orthochromic cells as giant orthochromic erythroblasts. Schwarz described binucleated and plurinucleated erythroblasts and pluripolar mitoses. His descriptions and illustrations clearly indicate the identity of these forms with the plurinuclear cells and pluripolar mitoses described by the other authors. In our material, all types of plurinuclear erythroblasts as well as those with lobulated nuclei described by the other authors are represented, although, as will be shown, examples of such cells at the proerythroblast or orthochromic levels are very rare in normal marrow.

\*The term erythroblast as used herein denotes any nucleated red cell which may undergo maturation changes from proerythroblast through basophilic, polychromatophilic, and orthochromic stages before final denucleation.

## MORPHOLOGY OF PLURINUCLEAR ERYTHROBLASTS AND PLURIPOLAR MITOSES AMONG ERYTHROBLASTS

Limarzi and Levinson described three main types of abnormal erythropoiesis. In the first type, a giant uninuclear erythroblast gave origin to plurinuclear cells by a series of multiple and complicated mitotic divisions without cytoplasmic division. The second type was characterized by a process of folding, indentation, lobulation, and constriction of the original nucleus so that eventually several

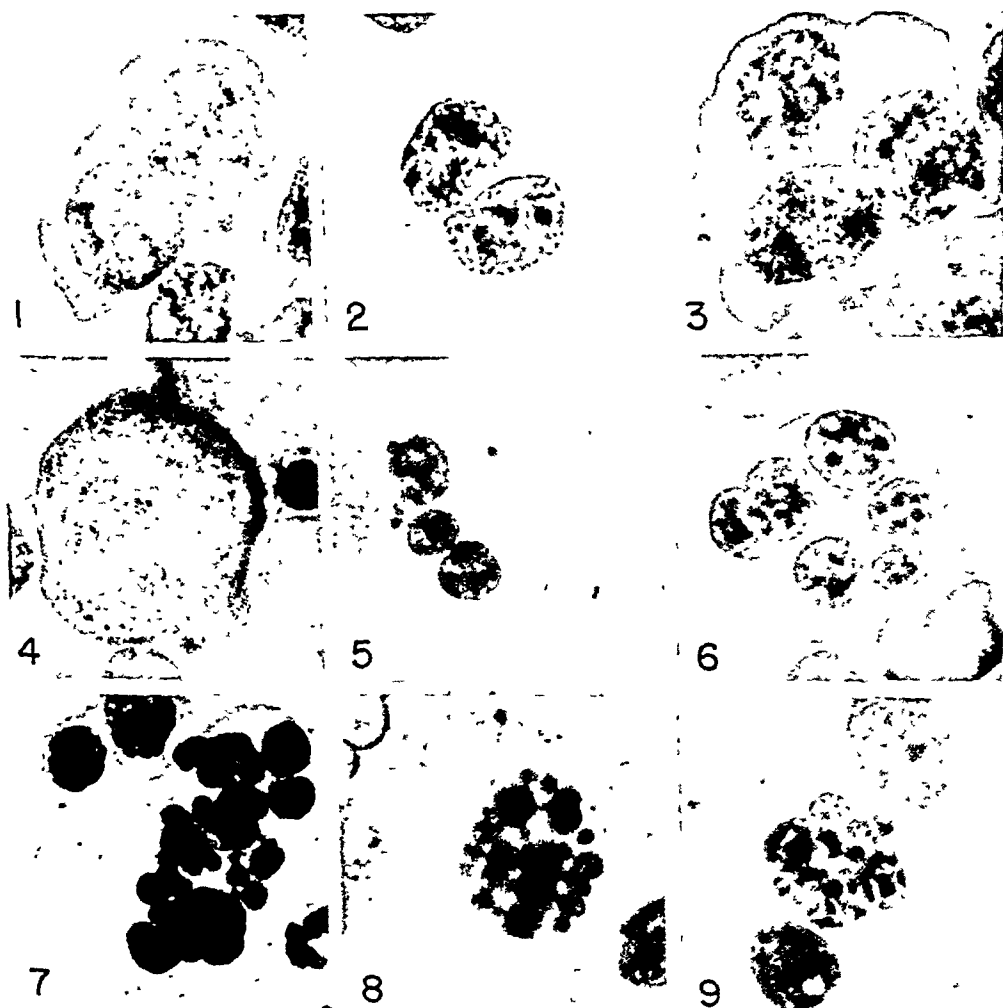


PLATE I

- 1.—Case 1. Binuclear proerythroblast 17 x 33 microns.
- 2.—Normal. Binuclear polychromatophilic erythroblast 21 x 29 microns.
- 3.—Case 1. Trinuclear early basophilic erythroblast 26 x 36 microns.
- 4.—Case 2. Trinuclear proerythroblast 25 x 27 microns.
- 5.—Case 1. Trinuclear erythroblast 18 x 27 microns.
- 6.—Case 2. Late erythroblast 21 x 26 microns.
- 7.—Case 2. Late erythroblast with many nuclear masses 21 x 36 microns.
- 8.—Case 1. Orthochromic erythroblast with many nuclear masses (karyorrhexis ?) 25 x 37 microns.
- 9.—Case 1. Late polychromatophilic erythroblast with fused or lobulated nuclei 20 x 30 microns.

independent nuclei were produced in a single cell. The third type was characterized by complete or incomplete complicated amitosis without cytoplasmic division. In Schwarz's material, examples of all these forms are described and illustrated. The giant orthochromic erythroblasts in Schleicher's material had diameters ranging from 15.8 to 39.6 microns. The nuclei were either lobulated, fragmented, or consisted of from five to eight deeply staining masses of irregular size.

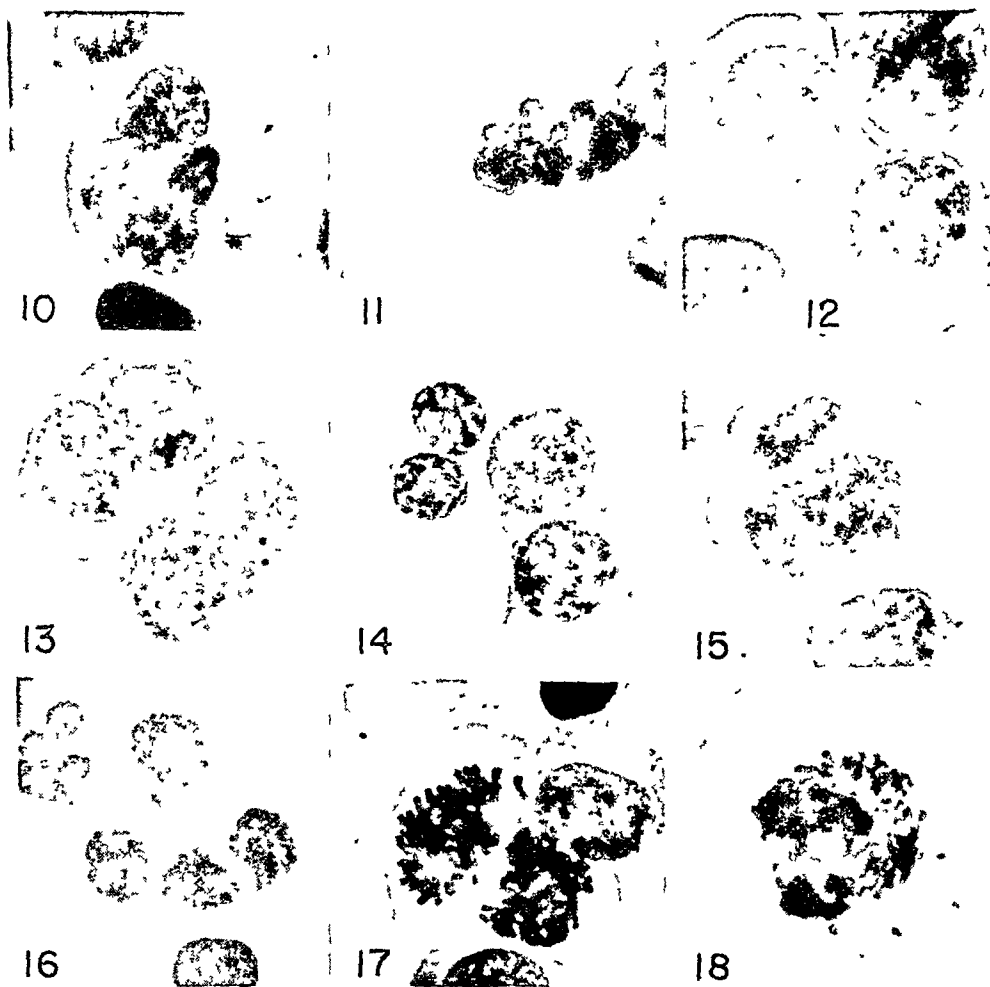


PLATE II

- 10—Case 1 Basophilic erythroblast with lobulated nucleus 17 x 28 microns  
 11—Case 1 Orthochromic erythroblast with lobulated nuclei and accessory nuclei masses (karyorrhexis?) 21 x 27 microns  
 12—Case 1 Incomplete cytokinesis in binuclear proerythroblast 17 x 40 microns  
 13—Case 2 Incomplete cytokinesis in quadrinuclear early basophilic erythroblast 25 x 37 microns  
 14—Case 2 Incomplete cytokinesis in two binuclear basophilic erythroblasts  
 15—Case 1 Tripolar mitosis with aberrant chromosomal mass in basophilic erythroblast 23 x 27 microns  
 16—Case 1 Quadripolar mitosis with aberrant chromosomal mass in polychromatophilic erythroblast 19 x 30 microns  
 17—Case 1 Irregular pluripolar mitosis in polychromatophilic erythroblast 20 x 27 microns  
 18—Case 1 Pluripolar mitosis with at least 7 asters in late polychromatophilic erythroblast 26 x 28 microns

The published detailed descriptions and illustrations of plurinuclear cells and pluripolar mitoses<sup>15, 17, 19, 20</sup> in both megaloblastic and nonmegaloblastic marrows are very complete and require little elaboration. In Plates I and II<sup>2</sup> are illustrated examples of such cells from one normal and three pathologic cases in our material. Examples from a case of pernicious anemia are omitted because of the general agreement as to their common occurrence in that disease. The identity of the cells illustrated and those represented by others is clear. It is of special interest that the condition of plurinuclearity in our material did not interfere with the relative progress of nuclear and cytoplasmic maturation. With very few exceptions, plurinuclear cells with basophilic erythroblastic nuclei were found to have cytoplasm characteristic of that maturation level. Similar normal relationships were observed in the polychromatophilic and orthochromic levels. These findings are in agreement with those of Schwarz whose study indicates that plurinuclearity does not interfere with the development and functional activities of the erythroblasts. Furthermore, the type of maturation and the morphologic forms of both nuclei and cytoplasm in the plurinuclear cells, in every instance, conformed to that of the prevailing type of erythropoiesis. In the normoblastic marrows (Cases 2, 4, 5, 6, 8, in addition to all normal cases) the plurinuclear cells had nuclear and cytoplasmic characteristics entirely similar to those of the remaining uninuclear normoblasts: in the megaloblastic marrow (Case 3), not only did the nuclear and cytoplasmic characteristics of the plurinuclear cells conform to that of the megaloblasts which predominated, but the relative rates of maturation of nuclei and hemoglobinization of cytoplasm were characteristic of the cells of the megaloblastic line, as described elsewhere.<sup>3</sup> In two instances (Cases 1 and 7) erythropoiesis involving the uninuclear erythroblasts could not be identified as either normoblastic or megaloblastic. The erythroblasts were classified as dysplastic, being somewhat intermediate in morphology between normoblasts and megaloblasts. In these marrows the plurinuclear erythroblasts and the remaining uninuclear erythroblasts had similar morphologic features. The sizes of the giants were approximate multiples of those of the corresponding stages of the uninuclear cells, roughly directly proportional to the amount of nuclear material, that is, the number of nuclei in each cell.

#### INCIDENCE OF PLURINUCLEAR ERYTHROBLASTS IN NORMAL HUMAN STERNAL MARROW

The literature does not contain statistical data concerning the incidence of plurinuclear erythroblasts or pluripolar mitoses among erythroblasts in normal human sternal marrow. Limarzi and Levinson stated that the occurrence of normal bipolar and pathologic multipolar mitosis of erythroid cells in the same marrow is an unusual finding in the adult. Schwarz stated that gigantism occurs in normal as well as in pathologic marrows, and also that he repeatedly encountered pluripolar mitoses in normoblastic regeneration, even in normal marrows. The myeloid:erythroid ratios and incidences of plurinuclear erythroblasts in normal individuals are shown in Table I.

\*The photomicrographs were prepared by Frank N. D. J. J.

TABLE I. MYELOID:ERYTHROID RATIOS AND INCIDENCE OF PLURINUCLEAR ERYTHROBLASTS IN STERNAL MARROW OF NORMAL INDIVIDUALS

CASE	AGE	M:E RATIO	PNE(0/100)	TOTAL ERB
1	18	3:1	5.0	13,065
2	22	2:1	2.0	5,010
3	24	2:1	2.2	5,011
4	30	2:1	1.2	5,006
5	31	2:1	1.0	5,005
6	36	2:1	2.8	5,014
7	45	2:1	1.0	5,005
8	53	2:1	5.1	10,051

M:E ratio, Myeloid: erythroid ratio.

PNE (%), number of plurinuclear erythroblasts per 1,000 uninuclear erythroblasts.

Total ERB, number of erythroblasts examined.

\*The myeloid: erythroid ratio represents the ratio of the number of leucocytes of myeloid origin, including monocytes, to the number of erythroblasts in the smears. It must be pointed out that the value is relative, not absolute. A decreased M:E ratio is indicative of a relative increase of erythropoiesis or relative decrease of myeloid leucopoiesis. The ratio cannot be used to determine absolute increase or decrease of erythropoiesis. However, our practice includes the preparation of gross marrow units which are evaluated by the method suggested by Schleicher.<sup>18</sup> In the presence of a cellular marrow with increased or normal frequency of erythroblastic islands, as determined by examination of the histologic pattern, a significantly lowered M:E ratio may be taken as a rough measure of the degree of acceleration or increase of erythropoiesis.

In the control series the incidences of plurinuclear erythroblasts vary from 1.0 to 5.1 per 1,000 uninuclear erythroblasts. The myeloid:erythroid ratios vary from 2:1 to 3:1. On the basis of the normal material available at present, it must be concluded that a myeloid:erythroid ratio significantly lower than 2:1 and an incidence of plurinuclear erythroblasts significantly greater than 5.1 per 1,000 uninuclear erythroblasts are to be regarded as abnormal. Furthermore, the presence of an appreciable number of pluripolar mitoses is abnormal, as indicated by the fact that in a total of 53,167 erythroblasts in eight normal persons, not a single example of pluripolar mitosis was seen. In addition, no examples of high plurinuclearity, that is, cells with more than two nuclei were encountered in normal marrows. Finally, the differential distribution of the plurinuclear erythroblasts, represented in Table II, reveals the relative rarity of plurinuclear pronormoblasts and plurinuclear orthochromic normoblasts. It is noteworthy that the differential distribution of the plurinuclear cells is similar to that of the normoblasts in general in normal individuals.

TABLE II. DIFFERENTIAL DISTRIBUTION OF PLURINUCLEAR AND UNINUCLEAR NORMOBLASTS IN NORMAL HUMAN STERNAL MARROW

	PNE	UNE
Total cells examined	167	2000
Pronormoblasts	1%	2%
Basophilic normoblasts	24%	11%
Polychromatophilic normoblasts	74%	86%
Orthochromic normoblasts	1%	1%

PNE, plurinuclear erythroblasts.

UNE, uninuclear erythroblasts.

One must conclude that the alleged rarity of pluripolar mitoses among erythroblasts in normal sternal marrow is an actuality, whereas the alleged rarity of plurinuclear erythroblasts must be explained on the basis of their having been overlooked in routine examination of marrow smears, due to their relatively small incidence.

INCIDENCE OF PLURINUCLEAR ERYTHROBLASTS AND PLURIPOLAR MITOSES AMONG ERYTHROBLASTS IN PATHOLOGIC HUMAN STERNAL MARROWS

Limarzi and Levinson present data indicating the incidence of cells classified as atypical erythroid cells, which we assume to represent the giant erythroblasts in their material, as 63.8 per 1,000 typical erythroblasts.\* Only one study was available during life. Schwarz does not offer statistical data regarding the incidence of plurinuclear cells. It is stated, however, that one case of myeloblastic leucemia was "quite outstanding in the degree and the frequency of the abnormality in question."<sup>20</sup> Schleicher gave the ratio of giant orthochromic erythroblasts to late pronormoblasts as 4 to 1 in one case, and he recorded the ratio of giant orthochromic erythroblasts to early and late basophilic erythroid cells as 3 to 1 in his second case. He did not discuss the significance of these ratios.

The incidences of plurinuclear erythroblasts in our pathologic marrows are shown in Table III. They vary from 16.0 to 159.6 per 1,000 uninuclear erythroblasts in the initial studies. Obviously, these data do not reflect the actual incidence of plurinuclear cells in pathologic marrows, since no attempt was made to survey all of our material. It can be stated, however, that rapid scanning of several slides with the low-power objective suffices for the discovery of such cells in bone marrow concentrate smears in which their frequency is in the neighborhood of 30 per thousand erythroblasts. They are easily noticed because of their large size. Plurinuclear erythroblasts easily may be overlooked in normal marrow smears because of their infrequent occurrence. On the other hand, it is difficult to understand how plurinuclear erythroblasts can be overlooked in pathologic marrows, especially in instances of increased erythropoiesis, since they are frequently present in our material and that of others.<sup>13, 20</sup> In Cases 1, 2, and 3 with incidences of 159.6, 99.2, and 52.8, respectively, the cells were so numerous that the smears presented a striking pathologic picture on

TABLE III. INCIDENCES OF PLURINUCLEAR ERYTHROBLASTS, MYELOID:ERYTHROID RATIOS, AND ERYTHROCYTE COUNTS IN PATHOLOGIC HUMAN STERNAL MARROWS

CASE	DIAGNOSIS		PNE(0/00)	M:E RATIO	RBC
1	Lymphoblastoma (?)	First study	159.6	1:1	1.84
		Second study	13.8	1:1	2.34
2	Chronic hemorrhage	First study	99.2	1:1	1.30
		Second study	5.4	1:2	1.40
3	Pernicious anemia	First study	52.8	1:2	0.73
		Second study	4.4	2:1	3.63
4	Hepatitis	First study	35.8	1:1	3.17
		Second study	6.0	3:1	4.00
5	Congenital hemolytic anemia	First study	32.0	1:2	1.13
		Second study	5.2	7:1	4.33
6	Cirrhosis of liver	First study	23.0	1:1	2.52
		Second study	6.0	7:1	3.80
7	Monocytic leucemia	First study	18.8	2:1	2.00
		Second study	22.0	2:1	2.21
8	Thrombocytopenic purpura	First study	16.0	1:2	2.26
		Second study	3.8	2:1	4.42

PNE(0/00), number of plurinuclear erythroblasts per 1,000 uninuclear erythroblasts.  
M:E ratio, myeloid:erythroid ratio.  
RBC, erythrocyte count in millions per cubic millimeter of blood.

\*Calculated from their data.

first inspection. The pathologic marrows also afforded excellent opportunities for examination of pluripolar mitoses (see individual case reports).

#### ON THE QUESTION OF THE TERMINAL NATURE OF THE PHENOMENON OF PLURINUCLEAR OF ERYTHROBLASTS

Schleicher's observation of two cases with giant orthochromic erythroblasts in patients with terminal illnesses, and Limarzi and Levinson's single case with terminal disease cannot be accepted as evidence of the terminal nature of the findings, since our series includes several cases showing an abnormal incidence of plurinuclear erythroblasts with clinical recovery (Table III). It is essential that serial marrow studies be made on patients exhibiting the change so that remission from the underlying clinical disease with a concomitant disappearance of the condition of plurinuclearity may not be overlooked.

#### ON THE QUESTION OF IRREVERSIBILITY AND CANCEROUS NATURE OF THE CHANGES

Some authors<sup>13, 19</sup> have considered the phenomenon under discussion to represent an irreversible change in the marrow or an indication of a defunct reticulum. As has been pointed out, the marrow pattern, in so far as erythropoiesis is concerned, may revert to normal after exhibiting a temporary period during which plurinuclear cells may be formed. The data already presented are evidence in favor of the concept of the reversibility of the lesion. The belief that the lesion is of cancerous nature<sup>15</sup> is partly based on the assumption that the changes are irreversible. Furthermore, it has been stated that bipolar mitosis and multipolar mitosis of erythroid cells in the same marrow is an unusual finding in the adult.<sup>15</sup> Our data do not conform to either of these assumptions. According to Schwarz, pluripolar mitosis does not represent a more fertile type of regeneration, as suggested by Limarzi, Jones, and Levinson,<sup>14</sup> nor is his material in accord with a biologic transformation akin to neoplastic deviation as assumed by these authors.

Although Schwarz does not present serial marrow studies on his cases showing plurinuclear erythroblasts, he concludes, largely on the basis of experimental evidence, that once normal conditions are restored the plurinuclear erythroblast tends to divide into as many daughter cells as there were nuclei formed by the preceding karyokinesis. The experimental work of Chambers,<sup>4</sup> cited by Schwarz as evidence for the reversibility of the phenomenon of suppressed cytokinesis, is based on the observations of sea-urchin eggs subjected to hypertonic sea water and later replaced in sea water, late amphister eggs placed in 2½ per cent ether solution in sea water and replaced within five minutes in sea water, late amphister eggs subjected to contact with 1½ to 2 per cent ether solutions in sea water or hypotonic solutions, and amphister eggs subjected to mechanical agitation. The following case reports are presented as evidence of reversibility of the changes under question in patients with clinical disease.

#### CASE REPORTS

CASE 1.—A white woman, aged 65 years, stated that she felt well until December, 1944, when she began to experience weakness, malaise, dyspnea, and weight loss. The only sig-

nificant findings on physical examination were enlarged liver and spleen. The edge of the liver was 5 cm. below the costal margin and the inferior border of the spleen extended below the umbilicus. On March 8, 1946, the initial blood studies showed: erythrocytes, 1.84 millions per cubic millimeter; hemoglobin, 7.7 grams per cent; reticulocytes, 1.5 per cent; leucocytes, 1,900 per cubic millimeter; platelets, 61,000 per cubic millimeter; mean corpuscular volume, 109 cubic microns; mean corpuscular hemoglobin, 42 micromicrograms. The differential distribution of the leucocytes was: band form neutrophils, 19 per cent; polymorphonuclear neutrophils, 21 per cent; monocytes, 23 per cent; lymphocytes, 38 per cent.

The bone marrow study revealed hypercellular marrow with marked increase in the frequency of erythroblastic islands as observed by inspection of gross marrow units (Schleicher<sup>18</sup>). The myeloid:erythroid ratio was 1:1. Erythropoiesis was atypical, characterized by the presence of dysplastic erythroblasts having a superficial resemblance to megaloblasts because of their large size and relatively vesicular chromatin networks in the nuclei. There were also numerous plurinuclear erythroblasts which were present in a frequency of 159.6 per thousand uninuclear erythroblasts (Table III). Myeloid leucopoiesis was relatively reduced, with band form neutrophils predominating. Megakaryocytopoiesis was reduced. There were 49 per cent lymphocytes. Numerous monocytoid cells recently derived from reticulum appeared in isolated instances and in syncytial masses with two to four nuclei. These resembled cells the author had previously encountered in imprints of lymph nodes from cases of reticulum cell type of lymphoblastoma, and they recall Schleicher's description of monocytoid reticulum cells in his Case 1, proved at autopsy to be reticulum cell sarcoma. The blood and marrow findings were summarized as macrocytic hyperchromic anemia, neutropenia, thrombocytopenia, probable reticulum cell type of lymphoblastoma with involvement of bone marrow, liver, and spleen, and accelerated dysplastic erythropoiesis with increased frequency of plurinuclear erythroblasts. In addition, there were numerous examples of pluripolar mitoses among erythroblasts (Plates I and II). Their incidence was 9.6 per thousand erythroblasts.

The patient received transfusions, vitamin B<sub>12</sub>, and oral folic acid. After twenty days, the appetite had returned and she began to gain weight and strength rapidly. On May 12, 1946, the blood study showed: erythrocytes, 2.11 millions per cubic millimeter; hemoglobin, 5.5 grams per cent; reticulocytes, 15.8 per cent; leucocytes, 3,000 per cubic millimeter; mean corpuscular volume, 107 cubic microns; mean corpuscular hemoglobin, 26 micromicrograms.

The bone marrow study revealed hypercellular marrow, as previously. The myeloid:erythroid ratio was 1:1. The most significant change from the previous myelogram was a reduction in the incidence of plurinuclear erythroblasts from 159.6 to 13.8 per 1,000 uninuclear erythroblasts (Table III). There were only 2.4 per 1,000 pluripolar mitoses in the second study.

*Comment.*—The case demonstrates the variability of the frequency of plurinuclear erythroblasts and pluripolar mitoses in a single patient. It is noteworthy that the reduction in their incidences occurred with improvement of the clinical condition of the patient and a reduction of the severity of the anemia. To be sure, the second study revealed that the changes were still present in a degree significantly greater than normal; but in view of the probability that the patient has a neoplastic disease affecting the liver, spleen, and bone marrow, the failure of the abnormality of erythropoiesis to disappear completely is to be expected. Furthermore, erythropoiesis was still accelerated at the second examination.

CASE 2.—A 52-year-old white man was admitted to the hospital June 3, 1946, with a history of dyspnea, weakness, tarry stools, and occasional vomiting. Three weeks before admission he drank an unknown quantity (approximately 2 pints) of a commercial shellac solvent. The significant findings were marked pallor, firm and barely palpable liver, and severe thrombosed and bleeding hemorrhoids. The serologic tests for syphilis were positive.



The stools revealed traces to 4 plus occult blood during the entire hospital stay. Repeated studies for ova and parasites gave negative results. Two x-ray examinations of the entire gastrointestinal tract failed to reveal any sources of the blood in the stools. The only known sources were hemorrhoids, which were very prominent. The patient refused surgical treatment. Biopsy of the liver showed a normal histologic structure. The initial blood study showed: erythrocytes, 1.30 millions per cubic millimeter; hemoglobin, 2.9 grams per cent; reticulocytes, 7.2 per cent; leucocytes, 6,500 per cubic millimeter; platelets, 100,000 per cubic millimeter; mean corpuscular volume, 77 cubic microns; mean corpuscular hemoglobin, 22 micromicrograms.

The bone marrow study revealed hypercellular marrow with marked increase of erythropoiesis and a myeloid:erythroid ratio of 1:1. Erythropoiesis was normoblastic. There were 99.2 plurinuclear erythroblasts and 3.1 pluripolar mitoses per 1,000 uninuclear normoblasts (Table III and Plates I and II). Myeloid leucopoiesis and megakaryocytopoiesis were unaltered. There were 13 per cent lymphocytes. The blood and marrow findings were summarized as microcytic hypochromic anemia, neutropenia, thrombocytopenia, and accelerated normoblastic erythropoiesis with increased frequency of plurinuclear erythroblasts.

During a period of observation from June 6 to 26, 1946, iron therapy and transfusions were withheld. On June 26 the blood study showed: erythrocytes, 1.40 millions per cubic millimeter.

The bone marrow findings were similar to those of the first study, with the exception that the incidence of plurinuclear cells had dropped from 99.2 to within a normal level of 5.4 per 1,000 uninuclear erythroblasts (Table III). No pluripolar mitoses were seen among 5,000 erythroblasts.

*Comment.*—The case illustrates a remission from the condition of atypical plurinuclear erythropoiesis, in spite of the continued presence of severe anemia and accelerated erythropoiesis.

CASE 3.—A 62-year-old white man with idiopathic pernicious anemia experienced an hematologic remission as a result of folic acid therapy. The initial blood study showed: erythrocytes, 730,000 per cubic millimeter; hemoglobin, 2.7 grams per cent; reticulocytes, 1.7 per cent; leucocytes, 5,800 per cubic millimeter; platelets, 16,000 per cubic millimeter; mean corpuscular volume, 122 cubic microns; mean corpuscular hemoglobin, 38 micromicrograms.

The bone marrow study revealed hypercellular marrow with increased erythropoiesis of megaloblastic type and a myeloid:erythroid ratio of 1:2. There were 52.8 plurinuclear erythroblasts and 2.8 pluripolar mitoses per 1,000 uninuclear erythroblasts (Table III). Only an occasional plurinuclear erythroblast had normoblastic character; the remaining plurinuclear erythroblasts were megaloblastic. Myeloid leucopoiesis revealed changes characteristic of antipernicious anemia principle deficiency.<sup>10</sup> Megakaryocytopoiesis was reduced.

At the time of the second marrow study, the blood showed: erythrocytes, 3.63 millions per cubic millimeter; hemoglobin, 10.7 grams per cent. The second bone marrow study revealed cellular marrow with slightly increased erythropoiesis and a myeloid:erythroid ratio within the normal range (2:1). Erythropoiesis was entirely normoblastic. The incidence of plurinuclear erythroblasts had dropped from 52.8 to 4.4 per 1,000 uninuclear erythroblasts (Table III). Only one pluripolar mitosis was found among 10,000 erythroblasts.

*Comment.*—The case illustrates the complete remission of the condition of excessive plurinuclearity and pluripolarity among erythroblasts in megaloblastic marrow as a result of specific therapy.

CASE 4.—A 54-year-old white man was admitted because of mild acute hepatitis. Serologic tests for leptospira were negative. Various liver function tests revealed only slight disturbances. The initial blood study on Oct. 16, 1946, showed: erythrocytes, 3.17 millions per cubic millimeter; hemoglobin, 11.6 grams per cent; reticulocytes, 6.6 per cent; leucocytes, 5,500 per cubic millimeter; platelets, 300,000 per cubic millimeter; mean corpuscular volume, 100 cubic microns; mean corpuscular hemoglobin, 37 micromicrograms.

The bone marrow study revealed slightly hypercellular marrow with increased erythropoiesis and a myeloid:erythroid ratio of 1:1. Erythropoiesis was normoblastic. There were 35.8 plurinuclear erythroblasts and 1.0 pluripolar mitoses per 1,000 uninuclear erythroblasts (Table III). The blood and marrow findings were summarized as macrocytic normochromic anemia and accelerated normoblastic erythropoiesis with increased frequency of plurinuclear erythroblasts.

The icterus cleared and the patient remained asymptomatic. A final blood study on Nov. 12, 1946, showed: erythrocytes, 4.00 millions per cubic millimeter; hemoglobin, 13.3 grams per cent; leucocytes, 7,150 per cubic millimeter.

The second bone marrow study revealed approximately normal cellularity and a myeloid:erythroid ratio of 3:1. The only significant finding in the myelogram was evidence of a decrease of the incidence of plurinuclear erythroblasts from 35.8 to 6.0 per 1,000 uninuclear cells (Table III). One pluripolar mitosis was seen.

*Comment.*—The case illustrates that the temporary condition of excessive plurinuclearity of erythroblasts may occur in relatively mild clinical disease.

CASE 5.—A white woman, aged 18 years, with congenital hemolytic anemia, was admitted because of an acute hemolytic episode. The initial blood study on Nov. 3, 1943, showed: erythrocytes, 1.13 millions per cubic millimeter; hemoglobin, 4.6 grams per cent; reticulocytes, 13 per cent; leucocytes, 26,400; platelets, 400,000 per cubic millimeter; mean corpuscular volume, 92 cubic microns; mean corpuscular hemoglobin, 41 micromicrograms. There were 60 normoblasts per 100 leucocytes. The erythrocytes were deeply staining cells of small diameter (spherocytes).

The bone marrow study revealed hyperplastic marrow with a myeloid:erythroid ratio of 1:2. Erythropoiesis was normoblastic. There were 32.0 plurinuclear erythroblasts and 1.8 pluripolar mitoses per 1,000 uninuclear erythroblasts (Table III). The blood and marrow findings were summarized as normocytic normochromic anemia with spherocytosis, normoblastic crisis, neutrophilic leucocytosis, and accelerated normoblastic erythropoiesis with increased frequency of plurinuclear erythroblasts. Splenectomy was performed Nov. 11, 1943, without complications.

At the time of the second marrow study on Nov. 23, 1943, the blood showed: erythrocytes, 4.33 millions per cubic millimeter; hemoglobin, 12.4 grams per cent. The second marrow study revealed hypercellular marrow with a myeloid:erythroid ratio of 7:1, revealing that the chief cause of the hypercellularity was increased leucopoiesis. The incidence of plurinuclear erythroblasts had fallen from 32.0 to 5.2 per 1,000 uninuclear erythroblasts (Table III), and only one pluripolar mitosis was seen among 7,000 erythroblasts.

*Comment.*—The case illustrates the remission of the condition of excessive plurinuclearity after removal of an important factor (hypersplenism) in the pathogenesis of the clinical disease.

CASE 6.—A 32-year-old white woman was admitted with cirrhosis of the liver, proved by biopsy. The initial blood study on June 16, 1946, showed: erythrocytes, 2.52 millions per cubic millimeter; hemoglobin, 9.4 grams per cent; reticulocytes, 2.4 per cent; leucocytes, 10,550 per cubic millimeter; platelets, 126,000 per cubic millimeter; mean corpuscular volume, 135 cubic microns; mean corpuscular hemoglobin, 37 micromicrograms.

The bone marrow study revealed hyperplastic marrow with a myeloid:erythroid ratio of 1:1. Erythropoiesis was normoblastic, with an incidence of 23.0 plurinuclear erythroblasts and 2.8 pluripolar mitoses per 1,000 uninuclear erythroblasts (Table III). The blood and marrow studies were summarized as macrocytic normochromic anemia, thrombocytopenia, and accelerated normoblastic erythropoiesis with increased frequency of plurinuclear erythroblasts.

The patient received various forms of therapy, following which her clinical condition improved. Four days before her discharge the blood study showed: erythrocytes, 3.88 millions per cubic millimeter; hemoglobin, 9.2 grams per cent.

The second marrow study revealed slightly hypercellular marrow without increased incidence of plurinuclear erythroblasts, which were now 6.0 per 1,000 uninuclear erythroblasts. There was only one pluripolar mitosis in approximately 12,500 erythroblasts.

*Comment.*—The case illustrates the disappearance of excessive plurinuclearity in a patient with a permanent lesion such as cirrhosis of the liver, concomitant with clinical improvement.

CASE 7.—A 27-year-old white man was admitted with monocytic leucemia. The initial blood studies showed: erythrocytes, 2.00 millions per cubic millimeter; hemoglobin, 4.5 grams per cent; reticulocytes, 0.1 per cent; leucocytes, 15,000; platelets, 30,000 per cubic millimeter; mean corpuscular volume, 75 cubic microns; mean corpuscular hemoglobin, 22.5 micromicrograms. The differential distribution of the leucocytes was: myeloblasts, 2 per cent; promyelocytes, 1 per cent; myelocytes, 1 per cent; metamyelocytes, 3 per cent; band form neutrophils 26 per cent; polymorphonuclear neutrophils, 14 per cent; eosinophile promyelocytes, 1 per cent; eosinophiles,  $\frac{1}{2}$  per cent; monocytes and immature monocytoïd cells, 26 per cent; lymphocytes, 25 per cent; plasma cells,  $\frac{1}{2}$  per cent; megakaryocytic nuclei, 1 per cent; atypical polychromatophilic erythroblasts, 3 per 100 leucocytes. There were numerous giant platelets.

The bone marrow study revealed hyperplastic marrow with a myeloid:erythroid ratio of 2:1. Erythropoiesis was of the dysplastic type, resembling that seen in Case 1. There were 18.8 plurinuclear erythroblasts, and an occasional pluripolar mitosis per 1,000 uninuclear erythroblasts (Table III). Myeloid leucopoiesis was altered because of the development of monocytoïd stem cells and monocytes, which comprised a total of 25 per cent of the nucleated cells. The hematologic findings were diagnostic of monocytic leucemia, Naegeli type. The patient received various forms of therapy, without effect.

Five months later, the marrow study revealed findings similar to those seen in the initial examination, with the exception that atypical stem cells and monocytoïd stem cells were more numerous and that the incidence of plurinuclear erythroblasts had increased slightly from 18.8 to 22.0 per 1,000 uninuclear erythroblasts.

*Comment.*—This case is included by way of contrast with the others, since it illustrates the persistence of the plurinuclear trait in a patient whose clinical condition became worse during the period of observation.

CASE 8.—A 23-year-old white woman with idiopathic thromboeytopenic purpura was admitted to the hospital because of approximately five months of purpura and bleeding from mouth and vagina. The initial blood study showed: erythrocytes, 2.26 millions per cubic millimeter; hemoglobin, 6.7 grams per cent; reticulocytes, 24.5 per cent; leucocytes, 12,400 per cubic millimeter; platelets, 1,900 per cubic millimeter; mean corpuscular volume, 93 cubic microns; mean corpuscular hemoglobin, 29.6 micromicrograms. There were 4 normoblasts per 100 leucocytes.

The bone marrow study revealed hyperplastic marrow with a myeloid:erythroid ratio of 1:2. Erythrocytogenesis was normoblastic, with 16.0 plurinuclear erythroblasts and 2.2 pluripolar mitoses per 1,000 uninuclear erythroblasts (Table III). Megakaryocyto-genesis was normal quantitatively. Splenectomy was performed without complications on Oct. 4, 1946.

All hemorrhagic and purpuric tendencies had disappeared by the fourth postoperative day. On Nov. 11, 1946, the blood showed: erythrocytes, 4.42 millions per cubic millimeter; platelets, 90,000 per cubic millimeter.

The second marrow study revealed approximately normal cellularity, with a myeloid:erythroid ratio of 2:1. The only other significant change in the myelogram was the appearance of evidence that the incidence of plurinuclear erythroblasts had dropped from 16.0 to 3.8 per 1,000 uninuclear erythroblasts. One pluripolar mitosis was seen among 5,000 erythroblasts.

*Comment.*—The case is an additional example of the disappearance of the condition of excessive plurinuclearity of erythroblasts concomitant with clinical and hematologic improvement following the removal of one of the important factors (hypersplenism) in the pathogenesis of the associated clinical disease.

#### DISCUSSION

The case reports presented include examples illustrating the temporary or reversible nature of the condition of plurinuclearity of erythroblasts and of pluripolarity of mitoses among erythroblasts in patients with either mild or serious clinical diseases (Cases 2, 3, 4, 5, 6, 8). The importance of such findings is obvious when applied to the concept of a cancerous nature of the changes. The fact that plurinuclear erythroblasts are present in normal marrows is additional evidence against their cancerous nature. Furthermore, the differential distributions and morphologic characters of the plurinuclear cells are remarkably similar to those of the uninuclear cells in the pathologic as well as the normal cases. This is suggestive of the action of the etiologic factors on any types of erythroblasts which are present at the time such factors come into play. Thus, the plurinuclear forms under question may be regarded as variants of the prevailing types of erythroblasts and not as members of a new genetic line of cells. In Cases 1 and 7 (lymphoblastoma and leucemia) with dysplastic erythropoiesis, the atypical giant plurinuclear cells could easily have been interpreted as representatives of a new cell line. However, the morphologic forms of the plurinuclear cells appeared to be modifications of the prevailing erythroblasts, readily explained by suppression of cytokinesis alone. It cannot be argued that because two of our cases occurred in association with malignant disease (Case 1, lymphoblastoma, and Case 2, leucemia), plurinuclearity of erythroblasts is related to malignant proliferation of cells in the bone marrow, since none of the other cases had any relationship to malignant disease. We have recently studied a case of polycythemia vera of several years' duration, now terminating as myelogenous leucemia. The bone marrow in this patient reveals only a normal incidence of plurinuclear cells.

A patient with chronic microcytic hypochromic anemia (Case 2) revealed a marked decrease in the incidence of plurinuclear erythroblasts to normal levels in spite of absence of clinical or hematologic improvement. While in most cases excessive plurinuclearity among erythroblasts was associated with accelerated erythropoiesis, it may be absent, or it may disappear, as in Case 2 in which a possible toxic agent (ingested shellac solvent) may have played an etiologic role.

The occurrence of the phenomena in relation to a progressive unfavorable course in patients who ultimately succumb to their clinical disease is not acceptable evidence in favor of an irreversible change. Indeed, the apparent nonreversibility is the result, not the cause, of the underlying clinical condition. Additional data may determine a range of incidences of plurinuclearity which is indicative of nonreversibility, but the present material shows that incidences of 99.2 and 159.6 per 1,000 are below such limits.

The present study does not disclose the action of factors common to all the cases. It is possible that when more cases illustrating plurinuclearity of high incidence are accumulated, such factors may be discovered. At any rate, the changes we have described, which involve suppression of cytokinesis without interference with maturation or karyokinesis, are not specific for any type or condition of erythropoiesis.

#### SUMMARY

The incidences of plurinuclearity and pluripolar mitoses among erythroblasts in sternal marrows of eight normal individuals have been determined to be 1.0 to 5.1, and less than 1.0 per 1,000 uninuclear erythroblasts, respectively.

The incidences of such changes in eight pathologic cases have been shown.

The reversible nature of the phenomena of plurinuclearity and pluripolarity among erythroblasts of normoblastic, dysplastic, and megaloblastic types has been demonstrated.

The significance of the findings has been discussed.

#### REFERENCES

1. Angelini, G., and Barasciutti, A.: *Morfologia delle cariocinesi nel midollo osseo*, Arch. per le sc. med. 66: 257-277, 1938 (cited by Schwarz<sup>20</sup>).
2. Berman, L., and Axelrod, A. R. A.: *Sternal Puncture Technique*, Am. J. Clin. Path. 17: 61-66, 1947.
3. Berman, L., Jacobson, S. D., and Vonderheide, E. C.: *Observations on Response of Macrocytic Anemias to Folic Acid Therapy*, Am. J. Clin. Path. 16: 61-66, 1947.
4. Chambers, R.: Section V. *Physical Structure of Protoplasm as Determined by Microdissection and Injection*, in Cowdry, E. V., editor: *General Cytology*, Chicago, 1924, University of Chicago Press, pp. 237-309.
5. Downey, H.: Cited by Limarzi and Levinson.<sup>15</sup>
6. Ehrlich, P., and Lazarus, A.: *Die Anämie*, Wien. 1898, Alfred Hölder.
7. Fieschi, A.: *Ricerche cariologiche sui megaloblasti*, Haematologica 18: 125-143, 1937.
8. Isaacs, R.: Cited by Limarzi and Levinson.<sup>15</sup>
9. Jacobsen, K. M.: *Untersuchungen über das Knochenmarkspunktat bei normalen Individuen verschiedener Altersklassen*, Acta med. Scandinav. 106: 417-446, 1941.
10. Jacobson, S. D., Berman, L., Axelrod, A. R., and Vonderheide, E. C.: *Neurological Exacerbations in Pernicious Anemia Patients Treated With Folic Acid*, J. A. M. A. In press.
11. Jones, O. P.: Cited by Limarzi and Levinson.<sup>15</sup>
12. Koller, F.: *Die Erythropoese bei der perniziösen Anämie mit besonderer Berücksichtigung der quantitativen Verhältnisse*, Deutsches Arch. f. klin. Med. 184: 568-626, 1939.
13. Leitner, S. J.: *Die intravitale Knochenmarksuntersuchung*, Basel, 1945, Benno Schwabe.
14. Limarzi, L. R., Jones, R. M., and Levinson, S. A.: *Unusual Erythropoiesis in Human Sternal Bone Marrow and Its Possible Relation to Megaloblastic Maturation*, Anat. Rec. 79: 43-44, 1941.
15. Limarzi, L. R., and Levinson, S. A.: *An Undescribed Type of Erythropoiesis Observed in Human Sternal Marrow*, Arch. Path. 36: 127-143, 1943.
16. Rohr, K.: *Das menschliche Knochenmark*, Leipzig, 1940, Georg Thieme.
17. Schilling, V.: *The Blood Picture* (translated and edited by Gradwohl, R. B. H.), St. Louis, 1929, C. V. Mosby Company.
18. Schleicher, E. M.: *The Volumetric Pattern of Aspirated Normal Human Sternal Marrow of Males 18 to 40 Years*, Am. J. Clin. Path. 14: 370-379, 1944.
19. Schleicher, E. M.: *Giant Orthochromic Erythroblasts*, J. Lab. & Clin. Med. 29: 127-133, 1944.
20. Schwarz, E.: *Cellular Gigantism and Pluripolar Mitosis in Human Hematopoiesis*, Am. J. Anat. 79: 75-116, 1946.
21. Segerdahl, E.: *Ueber Sternalpunktionen*, Acta med. Scandinav. supp. 64, pp. 1-162, 1935.

## ENHANCEMENT OF BLOOD LEVELS BY CARONAMIDE DURING INTRAMUSCULAR ADMINISTRATION OF PENICILLIN

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THE rapidity with which penicillin disappears from the blood and is excreted in the urine was recognized early in the study of this antibiotic.<sup>1,2</sup> Rammelkamp and Bradley,<sup>3</sup> on the basis of data showing that diodrast blocked the renal excretion of penicillin, suggested that penicillin was probably excreted by a tubular mechanism. Studies by Rantz and Kirby<sup>4</sup> demonstrated that in afebrile patients 750 to 1,120 ml. of plasma could be cleared of penicillin per minute. This was proof of the tubular excretion of penicillin since glomerular filtration could account for only a small fraction of such a large rate of excretion.

The possibility of prolonging the blood levels of penicillin by blocking the excretory channel has received considerable attention. Following the observation of Rammelkamp and Bradley referred to in the preceding paragraph, Beyer and associates<sup>5-7</sup> succeeded in decreasing the rate of excretion of penicillin by the use of para-aminohippuric acid. Several reports have appeared in which para-aminohippuric acid has been used with apparent success in clinical penicillin therapy.<sup>7-10</sup> Unfortunately, large amounts of diodrast or para-aminohippuric acid must be given to slow the excretion of penicillin significantly since these agents themselves are rapidly excreted by the renal tubules. The fact that both diodrast and para-aminohippuric acid must be administered intravenously is also a serious handicap to their general use.

Bronfenbrenner and Favour<sup>11</sup> found that the administration of 2.5 Gm. of benzoic acid by mouth every four hours to patients on an unrestricted diet may double the penicillin levels, and that the administration of this amount of benzoic acid to patients in whom fluid and salt intake is restricted may result in an even greater increase in the penicillin blood concentrations.

Recently Beyer<sup>12</sup> reported that the excretion of penicillin could be reversibly inhibited by 4'-carboxyphenylmethanesulfonanilide, a compound synthesized by Sprague and co-workers and called caronamide.<sup>13</sup> According to Beyer and co-workers,<sup>14</sup> this compound, while capable of blocking the tubular excretion of penicillin, is not itself subject to a significant degree of tubular excretion. Toxicity studies<sup>15</sup> showed caronamide to have an acute oral LD<sub>50</sub>

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of 1,405 mg. per kilogram for mice; 1,320 mg. per kilogram for rabbits; and 1,575 mg. per kilogram for dogs. No evidence of histologic damage or hematologic disturbances was observed in rats given 0.5 Gm. per kilogram of caronamide daily for four weeks or in dogs receiving daily doses as high as 1.5 Gm. per kilogram for the same length of time. Verwey and Miller<sup>16</sup> found that caronamide administered orally increased the effectiveness of a given intramuscular dose of penicillin against experimental infections in mice. They believe that the enhancement of penicillin activity by caronamide is due to the effect of the agent on the penicillin concentrations in the body rather than to any synergistic action.

Rapoport and associates<sup>17</sup> studied the toxicity of caronamide and its effectiveness in inhibiting penicillin excretion in fifteen children. In none of their patients did the drug have to be withdrawn because of toxicity when administered in oral doses of 0.1 to 0.4 Gm. per kilogram per day for one or two weeks. Three of their patients developed a rash. They found that 0.2 Gm. per kilogram per day of caronamide given orally increased the plasma levels of penicillin significantly, and that 0.4 Gm. per kilogram per day resulted in a marked rise in penicillin levels. No irreversible changes in glomerular filtration, renal plasma flow, maximum tubular excretory capacity (P. A. II. Tm), or maximum reabsorptive capacity (glucose Tm) were found.

Crosson and co-workers<sup>18</sup> in preliminary studies in adults found that 2 Gm. of caronamide given every four hours enhanced penicillin blood levels when the penicillin was given orally in a dose of 100,000 units or intramuscularly in a dose of 50,000 units. They found that administration of 1 Gm. of caronamide every two hours in similar subjects had little effect on the penicillin levels, while 1.5 Gm. every three hours definitely increased the penicillin levels. It is of interest that in the only patient to whom they gave 100,000 units of penicillin intramuscularly every four hours they failed to obtain increased penicillin levels with 2 Gm. of caronamide every four hours.

These findings seemed sufficiently encouraging to justify further study of caronamide, particularly in relation to the use of large doses of penicillin, with a view to the possibility of decreasing the frequency of injections while maintaining significant concentrations in the blood.

#### MATERIALS AND METHODS

*Choice of Subjects.*—The subjects were male hospital patients who were given penicillin, some of them during an acute illness but most of them during convalescence from various infections. Since it is known that advanced renal disease may interfere with penicillin excretion,<sup>2</sup> no patient was chosen in whom there was impairment of renal function as judged by the inability to concentrate urine or to maintain a normal concentration of nonprotein nitrogen in the blood. Otherwise the subjects were selected without regard to their disease. The patients were given the usual hospital diet and no restrictions were placed on their fluid or salt intake. Urine analyses and blood nonprotein nitrogen determinations were done before and during the caronamide administration.

*Penicillin and Caronamide Administration.*—All of the penicillin doses were given intramuscularly at eight-hour intervals. Crystalline sodium penicillin G, as available commercially, was used throughout and was given as a solution in normal saline in a concentration of 100,000 units per milliliter. The caronamide was supplied in 0.5 Gm. tablets and was given in various doses by mouth every four hours so that every other dose was given at the time of an intramuscular penicillin injection.

In the studies on the effect of caronamide on penicillin levels, the subjects were kept on a constant dosage of 100,000 units of penicillin every eight hours throughout the time when the caronamide was given, and for at least two days before the first dose, and for two, or more often three, days after the last dose of caronamide. The caronamide was usually given for twenty-four hours, and in a few instances during a forty-eight hour period, in order to permit the drug to reach an equilibrium in the body fluids.

*Penicillin Blood Levels.*—Blood for penicillin levels was obtained during the eight-hour intervals after the last dose of penicillin given during the period of caronamide administration. Two control series of blood were also obtained during similar eight-hour periods, one series just prior to the first dose, and the other two or three days after the last dose of caronamide. The blood was obtained just before and one-half, four, six, and eight hours after the penicillin injection. The first blood in each series was obtained, in each instance, at about 9 A.M. The concentrations of penicillin were determined on citrated plasma by the serial dilution method of Rammelkamp,<sup>19</sup> modified only by the use of 0.5 ml. amounts of the plasma dilutions.\*

## RESULTS

*Effect of 2 Gm. of Caronamide Every Four Hours.*—The plasma penicillin levels obtained in twelve subjects before, during, and after a period when caronamide was given in doses of 2 Gm. every four hours are shown in Table I. One-half of the subjects were under 60 years of age; in none of them was there any significant difference in the levels obtained while they received caronamide as compared with the control periods before and after the caronamide administration.

A marked effect from the caronamide was obtained in five of the six subjects over 60 years of age. The effect varied considerably in the different individuals. The levels obtained one-half hour after the dose of penicillin, which probably represent the maximum attained in most patients, were essentially the same (considering the limitations of the method) during the control periods and during the caronamide administration. At four and six hours after the penicillin injections, the levels obtained during the caronamide ingestion ranged from four to sixteen times those found during the control periods. At the beginning and end of the eight-hour interval, the levels in the

\*The end point was taken as the dilution in which there was no visible growth of the test organism in broth and on subculture to blood agar. In the occasional serum in which there was no visible growth in the broth and one or more colonies of streptococci developed on the blood agar, the level was taken as the mean of that concentration and the one in which no growth occurred on subculture.



TABLE I. PLASMA PENICILLIN LEVELS DURING INTRAMUSCULAR ADMINISTRATION OF 100,000 UNITS OF PENICILLIN EVERY EIGHT HOURS WITH AND WITHOUT ORAL CARONAMIDE, 2 GM. EVERY FOUR HOURS

SUBJECTS UNDER 60 YEARS OF AGE										SUBJECTS OVER 60 YEARS OF AGE									
CASE	AGE	CARON-AMIDE (GM. EVERY 4 HR.)	HOURS AFTER PENICILLIN DOSE							CASE	AGE	CARON-AMIDE (GM. EVERY 4 HR.)	HOURS AFTER PENICILLIN DOSE						
			0	1/2	4	6	8						0	1/2	4	6	8		
1	58	0	0*	2.0	0.03	0	0			7	70	0	0	1.5	0.03	0.01	0		
		2	0	1.0	0.03	0	0					2	0.12	2.0	0.5	0.25	0.12		
2	36	0	0	2.0	0.02	0	0			8	86	0	0	4.0	0.05	0	0		
		0	0	0.5	0	0	0					0	0.03	4.0	0.12	0.05	0.02		
		2	0	0.5	0.02	0	0					2	0.25	3.0	1.0	0.75	0.5		
3	44	0	0	0.25	0.02	0	0			9	69	0	0.02	1.0	0.12	0.03	0.03		
		0	0	0.5	0.02	0	0					0	0.02	1.0	0.03	0.06	0		
		2	0	1.0	0.02	0	0					2	0.03	1.0	0.12	0.06	0.03		
4	46	0	0	0.25	0	0	0			10	63	0	0	0.25	0.02	0.02	0.02		
		0	0	1.0	0.02	0	0					0	0	1.0	0.09	0.06	--		
		2	0	1.0	0.03	0	0					2	0.25	1.0	0.5	0.5	0.25		
5	46	0	0	0.5	0	0	0			11	73	0	--	--	--	--	--		
		0	0	2.0	0	0	0					0	0.06	2.0	0.18	0.12	--		
		2	0	1.0	0.02	0	0					2	0.5	4.0	1.0	1.0	0.5		
6	50	0	0	1.0	0	0	0					0	0.03	1.0	0.12	0.06	0.03		
		0	0	1.0	0.03	0.02	0			12	65	0	0	2.0	0.06	0.02	--		
		2	0.02	1.0	0.06	0.02	0					2	0.02	1.0	0.06	0.02	0		
		0	0	1.0	0.03	0	0					0	0	1.0	0.03	0.02	0		
Average		0	0	1.17	0.02	0	0			Average		0	0.02	1.92	0.09	0.05	0		
		2	0	0.92	0.03	0	0					2	0.20	2.0	0.53	0.43	0.23		
		0	0	0.83	0.01	0	0					0	0.01	1.45	0.07	0.02	0.02		

---, Not done.

\*All values &lt;0.01 unit are listed as 0.

same five subjects ranged from 0.03 to 0.5 unit per milliliter of plasma during the caronamide administration and from  $< 0.01$  to 0.06 unit during the control periods. The average increase in the levels for all the six subjects over 60 years of age was about six- and ninefold at four and six hours, respectively. The average level at the beginning and end of the eight-hour interval during caronamide administration was 0.20 and 0.23 unit per milliliter, respectively, as compared with 0.02 unit or less at the same times during the control periods.

*Effect of 3 Gm. of Caronamide Every Four Hours in Persons Under 60 Years of Age.*—The plasma penicillin levels obtained in four subjects under 60 years of age (Cases 13 to 16) who were given 3 Gm. of caronamide every four hours are shown in the upper part of Table II. In none of these four subjects was there any appreciable effect from the caronamide, the levels throughout the eight-hour period being essentially the same while the drug was given and during the control periods.

*Effect of 4 Gm. of Caronamide Every Four Hours in Subjects Under 60 Years of Age.*—Ten patients under 60 years of age (Cases 17 to 26) were given 4 Gm. of caronamide every four hours. The penicillin levels resulting from an intramuscular dose of 100,000 units of penicillin in these subjects before, during, and after the caronamide administration are also shown in Table II. An appreciable effect was obtained from the caronamide in every one of these ten subjects, but the effect varied widely in the different individuals. The levels obtained at one-half hour were higher during the caronamide administration in eight of the ten subjects. At four hours the levels during the caronamide period were from four to thirty-two times higher than at the same time during the control periods. At six hours only one subject failed to show an increased level; in the other nine the concentrations of penicillin were two to sixteen times higher during the caronamide administration. Demonstrable levels were obtained in most of the subjects at the end of eight hours (that is, just before the penicillin dose) while they were receiving caronamide but not during the control periods.

For the ten subjects under 60 years of age, the average plasma penicillin level eight hours after an intramuscular dose of 100,000 units of penicillin (or just before such a dose) during the oral administration of 4 Gm. of caronamide every four hours was 0.03 unit per milliliter. At six hours the average level was 0.08 unit per milliliter. Practically none was demonstrable at these times during the control periods in the same subjects. The average levels of penicillin one-half hour and four hours after the penicillin injections were, respectively, about two and ten times higher during the caronamide administration than they were during the control periods.

*Effect of Increasing the Dose of Penicillin.*—An attempt was made to estimate roughly the intramuscular dose of penicillin which, given at eight-hour intervals, would be required to maintain blood concentrations (particularly during the latter half of that interval) approximating those that resulted from injections of 100,000 units every eight hours during the oral administration of the minimum effective amounts of caronamide. For that purpose another group of subjects were given intramuscular doses of 100,000 units every eight

TABLE 11. PLASMA PENICILLIN LEVELS DURING INTRAMUSCULAR ADMINISTRATION OF 100,000 UNITS OF PENICILLIN EVERY EIGHT HOURS WITH AND WITHOUT CARONAMIDE, 3 OR 4 GM. EVERY FOUR HOURS (SUBJECT UNDER 60 YEARS OF AGE)

CASE	AGE	CARONAMIDE (GM. EVERY 4 HR.)	HOURS AFTER DOSE OF PENICILLIN				
			0	$\frac{1}{2}$	4	6	8
13	51	0	0*	1.0	0.02	0.02	0
		3	0	0.75	0.03	0.03	0.02
		0	0	1.0	0.03	0	0
14	42	0	--	--	--	--	--
		3	0	0.75	0.02	0	0
		0	0	1.0	0.02	0	0
15	31	0	0	0.5	0.02	0	0
		3	0	1.0	0.03	0	0
		0	0	0.5	0	0	0
16	14	0	0	1.5	0.02	0	0
		3	0	1.0	0.02	0	0
		0	0	1.0	0.02	0	0
Average		0	0	1.0	0.02	0.01	0
		3	0	0.88	0.03	0.01	0
		0	0	0.88	0.02	0	0
17	39	0	0	1.0	0.01	0	0
		4	0.03	2.0	0.12	0.03	0
		0	0	0.5	0	0	0
18	21	0	0	1.0	0	0	0
		4	0.03	2.0	0.12	0.03	0.02
		0	0	0.5	0	0	0
19	16	0	0	1.0	0.01	0	0
		4	0.06	2.0	0.25	0.06	0.03
		0	0	1.0	0.02	0	0
20	27	0	0	2.0	0.03	0.02	0
		4	0.02	2.0	0.25	0.12	0.03
		0	0	1.5	0.03	0.02	0
21	48	0	0	1.0	0.03	0	0
		4	0	2.0	0.12	0.06	0.02
		0	0	1.5	0.03	0.02	0
22	24	0	0	0.75	0.02	0.02	0
		4	0.03	4.0	0.06	0.02	0
		0	0	0.5	0.02	0	0
23	36	0	0	1.0	0.02	0	0
		4	0.06	1.0	0.25	0.12	0.06
		0	0	0.75	0.02	0	0
24	53	0	0	1.0	0.02	0	0
		4	0.02	2.0	0.06	0.02	0
		0	0	0.5	0.02	0	0
25	52	0	--	--	--	--	--
		4	0.03	1.0	0.25	0.12	0.06
		0	0	0.5	0.02	0	0
26	42	0	0.02	0.5	0.02	0	0
		4	0.06	4.0	0.5	0.25	0.06
		0	0	2.0	0.02	0	0
Average		0	0	1.03	0.02	0	0
		4	0.03	2.20	0.20	0.08	0.03
		0	0	0.93	0.02	0	0

--, Not done.

\* All values &lt;0.01 unit are listed as 0.

hours for one or two days. This was followed by 200,000 units every eight hours and finally 300,000 units every eight hours for similar periods. A series of blood penicillin levels were obtained during an eight-hour interval on each dose, just as in the previous studies.

Since it was shown that the blood levels on doses of 100,000 units were much better sustained in patients over 60 years of age than in those under

60 years, subjects of both age groups were chosen. The plasma penicillin levels obtained on these three dose levels in six subjects under 60 years and in a similar number of patients over 60 years of age are shown in Table III.

The expected increases in the penicillin levels with the progressive increases in the dose were noted, but there were considerable individual variations. Although marked differences were noted between the height of the levels obtained with any given dose in the two age groups, the increases in levels resulting from increasing the size of the dose were quite similar in both age groups.

*Comparison of Effects Obtained From Caronamide With Those Obtained From Increasing the Dose of Penicillin.*—In order to compare the effect of caronamide with that obtainable by increasing the dose of penicillin, it would be desirable to carry out all of the studies in the same subjects. This, however, was not feasible. A rough estimate of the comparative effects may nevertheless be made from the data already presented. For that purpose it is best to consider the subjects in the two age groups (that is, those under 60 years of age and those over 60 years of age) separately.

The comparison in the subjects over 60 years of age may be made from the data shown for Cases 7 to 12 in Table I and for Cases 33 to 38 in Table III, particularly from the average values given in these tables. A similar comparison in the subjects under 60 years of age may be made from the levels shown in Table II (Cases 17 to 26) for the effects of 4 Gm. of caronamide and those given for Cases 27 to 32 in Table III. The average values for these groups are shown graphically in Fig. 1. In this figure the averages for the control levels obtained before and after caronamide are included in a single curve.

The curves representing the average levels following increasing doses of penicillin were obtained in the same patients. Those representing the effect of caronamide, however, are averages from other groups of patients. It should be noted that all of the latter patients had been on caronamide therapy for at least sixteen hours before the penicillin levels were determined so that there were appreciable penicillin serum concentrations at zero hour on these curves.

The difference in the declining slope of the curves between the patients under and those over 60 years of age is striking. In the older patients a significant concentration of penicillin persisted in the serum eight hours after the injection of 100,000 units, even when caronamide was not given, whereas in the younger subjects little or no penicillin was detectable six hours after such an injection. Increasing the dose of penicillin altered the height of the curves to a similar extent in both age groups but did not significantly alter the slope of these curves. The caronamide, on the other hand, increased the peak penicillin levels to some extent, but its effect on slowing the rate of fall of the plasma penicillin concentrations was striking. Except at the time of the peaks, the patients receiving the caronamide and 100,000 units of penicillin had significantly higher levels than those receiving 300,000 units of penicillin without caronamide. This difference was greatest in both age groups six

TABLE III. PLASMA PENICILLIN LEVELS DURING INTRAMUSCULAR ADMINISTRATION OF INCREASING AMOUNTS OF PENICILLIN GIVEN EVERY EIGHT HOURS WITHOUT CARONAMIDE

SUBJECTS UNDER 60 YEARS OF AGE										SUBJECTS OVER 60 YEARS OF AGE									
CASE	AGE	PENICILLIN (UNITS EVERY 8 HR.)		HOURS AFTER PENICILLIN DOSE						CASE	AGE	PENICILLIN (UNITS EVERY 8 HR.)		HOURS AFTER PENICILLIN DOSE					
27	36	100,000	0*	1.0	0.02	0	0	0	0	33	60	100,000	0.01	2.0	0.06	0	0	0	0
		200,000	0	2.0	0.03	0	0	0	0			200,000	0	4.0	0.12	0.03	0.02	0.02	0.02
		300,000	0	4.0	0.06	0.02	0.02	0.02	0.02			300,000	0.02	4.0	0.25	0.03	0.02	0.02	0.02
28	39	100,000	0	0.5	0.02	0	0	0	0	34	77	100,000	0.02	1.0	0.12	0.02	0.02	0.02	0.02
		200,000	0	2.0	0.06	0.02	0	0	0			200,000	0	4.0	0.25	0.12	0.03	0.03	0.03
		300,000	0.02	--	0.12	0.03	0.03	0.03	0.03			300,000	0.12	8.0	0.5	0.12	0.06	0.06	0.06
29	57	100,000	0	2.0	0.03	0.02	0	0	0	35	73	100,000	0.12	2.0	0.5	0.12	0.12	0.12	0.12
		200,000	0	2.0	0.03	0.02	0.02	0.02	0.02			200,000	0.25	2.0	0.8	0.5	0.25	0.25	0.25
		300,000	0.02	6.0	0.25	0.06	0.06	0.06	0.06			300,000	0.25	4.0	1.0	0.5	0.25	0.25	0.25
30	52	100,000	0	0.75	0.03	0	0	0	0	36	79	100,000	0.03	2.0	0.12	0.03	0.02	0.02	0.02
		200,000	0	2.0	0.09	0.02	0	0	0			200,000	0.03	2.0	0.18	0.06	0.03	0.03	0.03
		300,000	0	8.0	0.12	0.03	0.03	0.03	0.03			300,000	--	--	--	--	--	--	--
31	14	100,000	0	2.0	0.02	0	0	0	0	37	74	100,000	0	1.0	0.06	0	0	0	0
		200,000	0.02	2.0	0.02	0	0	0	0			200,000	0	2.0	0.06	0.02	0.02	0.02	0.02
		300,000	0	8.0	0.03	0	0	0	0			300,000	0.02	4.0	0.5	0.06	0.06	0.06	0.06
32	17	100,000	0	2.0	0.05	0.02	0	0	0	38	78	100,000	0	1.0	0.25	0.03	0.03	0.03	0.03
		200,000	--	4.0	0.12	0.03	0.02	0.02	0.02			200,000	0.03	2.0	0.25	0.06	0.06	0.06	0.06
		300,000	0.02	8.0	0.25	0.03	0.03	0.03	0.03			300,000	0.06	4.0	0.5	0.25	0.25	0.25	0.25
Average		100,000	0	1.38	0.03	0.01	0	0	0	Average		100,000	0.03	2.0	0.19	0.03	0.03	0.03	0.03
		200,000	0	2.33	0.10	0.02	0.01	0.01	0.01			200,000	0.05	2.67	0.28	0.13	0.13	0.13	0.13
		300,000	0.01	6.80	0.14	0.03	0.03	0.03	0.03			300,000	0.09	4.8	0.55	0.19	0.19	0.19	0.19

-- Not done.

\*All values &lt;0.01 unit are listed as 0.

hours after the penicillin injection. The levels obtained with 100,000 units of penicillin (without caronamide) were comparable within each age group in both series of studies.

*Toxicity.*—There was no clinical or laboratory evidence of toxicity from the caronamide in any of the subjects. In no instance was crystalluria, hematuria, or albuminuria observed which could be attributed to caronamide. No

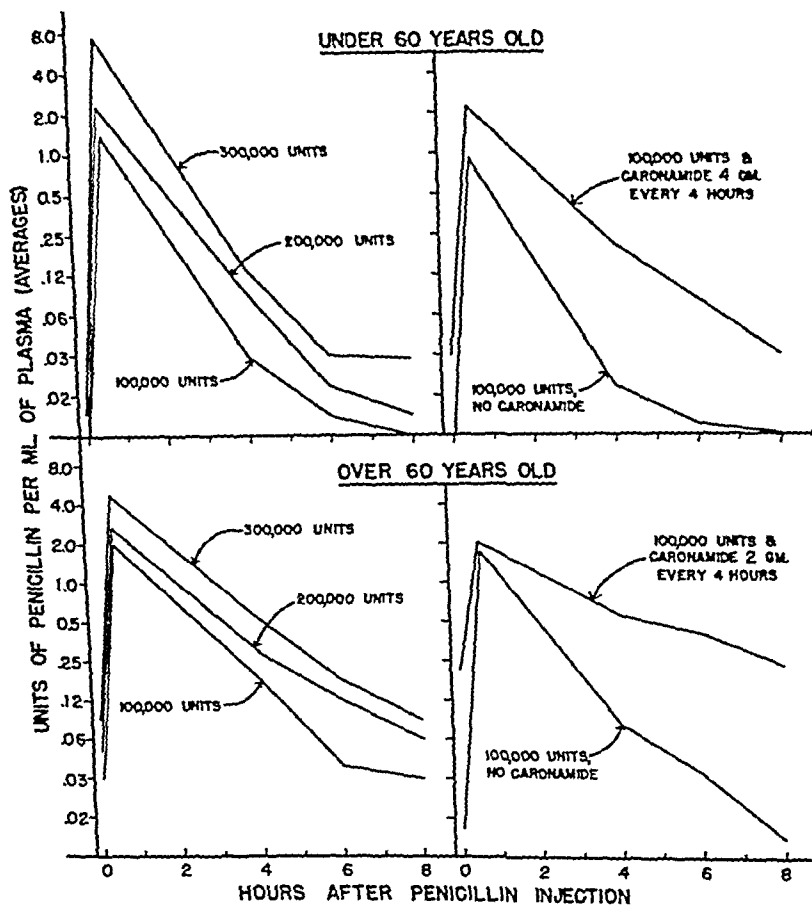


Fig. 1.—Enhancement of plasma levels by caronamide during intramuscular administration of 100,000 units of penicillin every eight hours; comparison with effect of increasing the dose in subjects within the same age group.

significant increase in the level of blood nonprotein nitrogen occurred. Furthermore, the reducing substance reported by Beyer and co-workers<sup>15</sup> as occurring in the urine of animals during administration of caronamide in large doses was not encountered in any of these cases. The findings in the present cases, however, must be interpreted in the light of the brief period during which the caronamide was given.

#### COMMENT

These studies confirm the findings previously mentioned and show conclusively that caronamide administered by mouth in adequate doses signifi-

cantly enhances penicillin blood levels. Crosson and associates<sup>18</sup> reported that 2 Gm. of caronamide did not have an appreciable effect on the penicillin levels in one of their patients who was receiving 100,000 units of penicillin intramuscularly, whereas the dose was effective in other patients receiving 50,000 units of penicillin. While the age of their patient is not given, the data presented in the foregoing show that 4 Gm. of caronamide is necessary to enhance penicillin levels in persons under 60 years of age who are receiving 100,000 units of penicillin intramuscularly every eight hours and have reasonably good renal function.

Rapoport and co-workers<sup>17</sup> noted significant increases in penicillin blood levels in children with the use of 0.2 Gm. of caronamide per kilogram per day. In adults averaging 60 kilograms in weight, the corresponding dose (namely, 12 Gm. per day) was found adequate for the purpose of increasing the blood levels from the intramuscular dose of penicillin used here only in subjects over 60 years of age. In younger adults twice this amount was necessary to obtain a similar result.

The relative effect of any given dose of caronamide on the blood levels obtained from varying doses of penicillin was not studied.

A comparison of the curves in Fig. 1 shows that the rate of disappearance of penicillin from the blood is significantly slower in the patients over 60 years than in the younger subjects. Since penicillin is excreted chiefly by the tubules, even a major diminution in glomerular filtration could not explain the difference in excretion between the younger and older groups of patients. Presumably, therefore, the reduced rate of disappearance of penicillin from the blood of the older patients is due to a reduction in their functional tubular mass.

Examination of Fig. 1 also shows that the administration of caronamide alters the penicillin blood level curve in young people in a manner qualitatively similar to the alteration apparently caused by age alone. Furthermore, the fact that less caronamide is required to enhance the penicillin levels significantly in the older patients is further evidence of reduced tubular excretory function in the older people.

#### SUMMARY AND CONCLUSIONS

Caronamide administered orally in doses of 2 Gm. every four hours to patients over 60 years of age markedly enhanced the plasma penicillin levels resulting from the intramuscular administration of 100,000 units of penicillin every eight hours. It was necessary to increase the caronamide dosage to 4 Gm. every four hours in order to produce a similar effect in younger persons. The penicillin blood levels following intramuscular administration of 100,000 units of penicillin every eight hours to patients receiving approximately minimal effective oral doses of caronamide were significantly higher than those obtained by giving 300,000 units of penicillin alone to similar subjects.

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## REFERENCES

1. Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Bentley, N. G., Jennings, M. A., and Florey, H. W.: Further Observations on Penicillin, *Lancet* 2: 177, 1941.
2. Rammelkamp, C. H., and Keefer, C. S.: The Absorption, Excretion and Distribution of Penicillin, *J. Clin. Investigation* 22: 425, 1943.
3. Rammelkamp, C. H., and Bradley, S. E.: Excretion of Penicillin in Man, *Proc. Soc. Exper. Biol. & Med.* 53: 30, 1943.
4. Rantz, L. A., and Kirby, W. M. M.: The Absorption and Excretion of Penicillin Following Continuous Intravenous and Subcutaneous Administration, *J. Clin. Investigation* 23: 789, 1944.
5. Beyer, K. H., Woodward, R., Peters, L., Verwey, W. F., and Mattis, P. A.: The Prolongation of Penicillin Retention in the Body by Means of Para-aminohippuric Acid, *Science* 100: 107, 1944.
6. Beyer, K. H., Peters, L., Woodward, R., and Verwey, W. F.: The Enhancement of the Physiological Economy of Penicillin in Dogs by the Simultaneous Administration of Para-aminohippuric Acid, *J. Pharmacol. & Exper. Therap.* 82: 310, 1944.
7. Beyer, K. H., Flippin, H., Verwey, W. F., and Woodward, R.: The Effect of Para-aminohippuric Acid on Plasma Concentrations of Penicillin in Man, *J. A. M. A.* 126: 1007, 1944.
8. Loewe, L., Rosenblatt, P., Altire-Werber, E., and Kozak, M.: The Prolonging Action of Penicillin by Para-aminohippuric Acid, *Proc. Soc. Exper. Biol. & Med.* 58: 298, 1945.
9. Mokotoff, R., Brams, W., Katz, L. N., and Howell, K. M.: Treatment of Bacterial Endocarditis With Penicillin; Results of 17 Consecutive Unselected Cases, *Am. J. M. Sc.* 211: 395, 1946.
10. Loewe, L., Rosenblatt, P., and Altire-Werber, E.: A Refractory Case of Subacute Endocarditis Due to *Feillonella gazogenes* Clinically Arrested by a Combination of Penicillin, Sodium Para-aminohippurate, and Heparin, *Am. Heart J.* 32: 327, 1946.
11. Bronfenbrenner, J., and Favour, C. B.: Increasing and Prolonging Blood Penicillin Concentrations Following Intramuscular Administration, *Science* 101: 673, 1945.
12. Beyer, K. H.: New Concept of Competitive Inhibition of the Renal Tubular Excretion of Penicillin, *Science* 105: 94, 1947.
13. Sprague, J. M., Ziegler, C., Cragoe, E. J., and Miller, C. S.: 4'-Carboxyphenylmethanesulfonanilide. To be published.
14. Beyer, K. H., Russo, H. F., Patch, E. A., and Tillson, E. A.: Caronamide: Its Pharmacologic Properties. To be published.
15. Beyer, K. H., McKinney, S. E., Tillson, E. A., and Green, C. W.: Caronamide: Its Toxicologic Effects. To be published.
16. Verwey, W. F., and Miller, A. K.: The Effect of Caronamide Upon Penicillin Therapy of Experimental Pneumococcus and Typhoid Infections in Mice. To be published.
17. Rapoport, M., Beyer, K. H., Corneal, M. B., and Verwey, W. F.: A Clinical Evaluation of the Toxicity and Efficacy of Caronamide for the Competitive Inhibition of Penicillin Excretion. To be published.
18. Crosson, J. W., Boger, W. P., Shaw, C. C., and Miller, K. A.: Enhancement of Penicillin Plasma Concentration With Caronamide. To be published.
19. Rammelkamp, C. H.: A Method for Determining the Concentration of Penicillin in Body Fluids and Exudates, *Proc. Soc. Exper. Biol. & Med.* 51: 95, 1942.



# ENHANCEMENT OF PLASMA PENICILLIN CONCENTRATIONS BY CARONAMIDE AND SODIUM BENZOATE

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## INTRODUCTION

A CONSIDERABLE number of drugs and vehicles have been introduced into clinical practice in an attempt to improve the practical therapeutics of penicillin by enhancing and prolonging its effective concentration in the blood. Among other agents, benzoic acid and sodium benzoate have been used rather widely to elevate and prolong plasma penicillin concentrations. Despite their use, however, relatively little data are available with regard to their efficacy. Bronfenbrenner and Favour<sup>1</sup> found that blood penicillin concentrations were doubled when benzoic acid was also administered. Bohls and co-workers<sup>2, a, b</sup> reported measurable blood concentrations of penicillin for twenty-four or more hours following both intramuscular and oral administration of an aluminum-penicillin mixture with benzoic acid or sodium benzoate. These results were not confirmed by Spaulding and associates,<sup>3</sup> who found only a moderate enhancement of plasma penicillin concentrations, in some instances for six hours, following the oral administration of several preparations of penicillin with sodium benzoate.

Beyer and associates have introduced a new substance, caronamide, which was found to be effective in enhancing and prolonging penicillin plasma concentrations.<sup>4</sup> Caronamide (4'-carboxy-phenylmethanesulfonanilide) is a stable, crystalline, colorless compound with little or no odor or taste, only slightly soluble in water, and relatively stable to hydrolysis with acids or alkali. In renal clearance studies in dogs it was demonstrated that caronamide decreased tubular excretion and increased the plasma concentration of penicillin.<sup>5</sup> When caronamide was given in a dosage of 50 mg. per kilogram the renal clearance of penicillin approximated that of creatinine, indicating an essentially complete suppression of tubular excretion of penicillin. This effect, which was reversible, persisted for about four hours. Further renal clearance studies indicated that caronamide did not influence the Tm of glucose or arginine, or the clearances of urea, sulfonamides, or creatinine.<sup>6</sup> As would be predicted, however, it decreased the clearance of para-aminohippuric acid. The mechanism of action of caronamide appears to stand in sharp contrast to para-aminohippurate and diodrast. The latter drugs depress penicillin excretion by a mass competition or blockade phenomenon.<sup>7, 8</sup> Caronamide, in contrast, is believed to be excreted wholly through the glomerular filtrate. Because caronamide depressed the tubular excretion of penicillin but was not itself excreted

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through the tubules. Beyer postulated that this substance exerts a selective inhibitory effect on the enzymatic mechanism of the renal tubules which effects the elimination of penicillin.<sup>4</sup>

Caronamide was found to exhibit a low order of both acute and chronic toxicity in laboratory animals.<sup>9</sup> When the drug was given to children for periods of one to two weeks, serious toxic reactions were not observed. The oral administration of caronamide and penicillin resulted in an increase in plasma penicillin concentrations of from 1.8- to 14.5-fold over the concentrations obtained during control periods.<sup>10</sup> Similar results with caronamide were also observed in adults who received penicillin intramuscularly and orally.<sup>11</sup>

These results appeared to be of sufficient interest to warrant the employment of caronamide under clinical conditions. Approximately thirty-five subjects received caronamide by mouth together with penicillin (1) by mouth, (2) by intramuscular injection in aqueous solution, and (3) by intramuscular injection in peanut oil and beeswax. The effect of caronamide on the plasma concentration of streptomycin was also studied in four individuals.

When it became apparent that caronamide did have an effect on the plasma penicillin concentration, the study was extended to include a comparison of the effectiveness of caronamide and sodium benzoate, individually and in combination, in eight additional subjects.

#### MATERIALS AND METHODS

The subjects used were all young adults and were either healthy physicians, medical students, and nurses or patients convalescent from a variety of illnesses. The twelve subjects who received penicillin in peanut oil and beeswax had primary or secondary syphilis. None of the subjects had clinical or laboratory evidence of renal disease. All ate a general diet and most of them were ambulatory.

Caronamide\* was supplied in tablet form and taken by mouth with water. Individual doses varied from 1½ to 3 Gm. at intervals of two to three hours, as indicated subsequently. Sodium benzoate was put up in soft gelatin capsules and taken by mouth with water, in 2 Gm. doses. The sodium salt of amorphous penicillin was used for intramuscular injection. Penicillin in peanut oil and beeswax was supplied as the calcium salt of amorphous penicillin and contained 300,000 units per milliliter. The preparation of penicillin for oral administration was in the form of tablets, each containing 50,000 units of potassium penicillin G with aluminum hydroxide.\*

In all of the studies, each subject served as his own control. On the first day of the test penicillin was given alone, at the intervals and in the dosages indicated subsequently. On the following day, the same dosage schedule of penicillin was resumed and, in addition, either caronamide or sodium benzoate, or both, were administered. Oxalated blood samples were obtained at the same hours on each day of the study.

An attempt was made to administer the drugs between meals, allowing an hour between medication and the ingestion of food. Subjects were asked to

\*Supplied by Dr. J. W. Crosson, of Sharp & Dohme, Inc., Philadelphia, Pa.

maintain the same diet and fluid intake on each day of the test, but no restrictions were imposed on salt or water intake. Close control of the schedules of meals and medication were not always possible, and it is not known, in some instances, how carefully the instructions were followed.

Plasma penicillin concentrations were determined by a modification of the serial dilution method of Rammelkamp.<sup>12</sup> The medium used was brain-heart infusion broth.\* The test organism was a hemolytic streptococcus (strain No. 98†) which was inhibited by 0.008 unit of penicillin per milliliter of broth. The end points of the plasma specimens were determined by the presence or absence of growth of explants on blood agar from the tubes with the highest dilutions of plasma which were clear after twenty-four hours' incubation. Despite the recognized inaccuracies of the method, replicate determinations on the same samples yielded the same results. The method is believed accurate for the detection of concentrations of 0.03 unit or more of penicillin per milliliter of plasma.

Caronamide and sodium benzoate in concentrations of 1 mg. per milliliter of broth did not inhibit the growth of the test organism. These concentrations of the drugs, alone and in combination, did not interfere with the in vitro activity of penicillin, in either broth or diluted plasma. Moreover, the plasma obtained from an individual one and one-half hours after the ingestion of 3.0 Gm. of caronamide did not affect the activity of penicillin in vitro. It was concluded, therefore, that the effects of these agents on plasma penicillin concentrations were not due to interference with the sensitivity of the penicillin assay procedure itself.

## RESULTS

### *Effect of Caronamide.—*

*Penicillin Administered by Mouth:* The effect of caronamide on orally administered penicillin was studied in seventeen tests performed on thirteen subjects. In twelve tests (ten subjects) 100,000 units of penicillin were taken by mouth at two-hour intervals from 8 A.M. to 4 P.M. On the next day, the same dosage of penicillin was resumed and, in addition, the subjects received 2 Gm. of caronamide by mouth every two hours from 6 A.M. to 4 P.M. In six tests blood samples were obtained one hour after each dose of penicillin on both days of the tests; in the other six tests the blood samples were obtained two hours after each dose of penicillin and immediately preceding the next dose. The resulting plasma penicillin concentrations are shown in Tables I and II. There can be little doubt that when these subjects received caronamide, the concentrations of penicillin in the plasma were increased as compared with the control periods. In twenty-five paired tests with blood obtained one hour after each dose of penicillin, the plasma concentrations of penicillin were the same with and without caronamide in three, lower with caronamide only once, and higher with caronamide in twenty-one tests. The increase in penicillin concentration associated with the administration of caronamide was twofold in nine, fourfold in five, and eightfold or more in seven

\*Difco Laboratories, Inc., Detroit, Mich.

†Obtained from Dr. M. Finland.

TABLE I. EFFECT OF CARONAMIDE ON PLASMA PENICILLIN CONCENTRATION  
(PENICILLIN: 100,000 UNITS, BY MOUTH, EVERY TWO HOURS;  
BLOOD SAMPLES, ONE HOUR AFTER EACH DOSE)

SUBJECT	SEX	DRUG	PLASMA PENICILLIN CONCENTRATION (UNITS/ML.)				
			9 A.M.	11 A.M.	1 P.M.	3 P.M.	5 P.M.
P. R.	M	Penicillin alone + Caronamide	<0.03 <0.03	<0.03 <0.03	0.22 0.06	0.06 0.11	0.03 0.06
P. R.	M	Penicillin alone + Caronamide	---	0.03 0.11	0.03 0.06	0.03 0.06	0.03 0.11
P. S.	M	Penicillin alone + Caronamide	0.03 0.03	0.06 0.11	0.03 0.22	0.03 0.22	<0.03 0.45
P. S.	M	Penicillin alone + Caronamide	---	0.03 0.06	0.11 0.22	0.06 0.11	0.03 0.45
N. W.	F	Penicillin alone + Caronamide	---	0.11 0.45	0.03 0.11	0.11 0.22	---
N. P.	F	Penicillin alone + Caronamide	---	0.03 0.45	0.03 0.45	0.11 0.45	0.06 0.45

Caronamide (2 Gm. P.O.) at 6, 8, and 10 A.M., 12 N., and 2 and 4 P.M.

Penicillin (100,000 units P.O.) at 8 and 10 A.M., 12 N., and 2 and 4 P.M.

---, Blood sample not obtained.

TABLE II. EFFECT OF CARONAMIDE ON PLASMA PENICILLIN CONCENTRATION  
(PENICILLIN: 100,000 UNITS, BY MOUTH, EVERY TWO HOURS;  
BLOOD SAMPLES, TWO HOURS AFTER EACH DOSE)

SUBJECT	SEX	DRUG	PLASMA PENICILLIN CONCENTRATION (UNITS/ML.)			
			10 A.M.	12 N.	2 P.M.	4 P.M.
J. L.	M	Penicillin alone + Caronamide	0.03 0.03	0.06 0.11	0.03 0.22	0.06 0.22
N. M.	F	Penicillin alone + Caronamide	0.03 0.11	0.11 0.22	0.03 0.22	0.06 0.11
N. N.	F	Penicillin alone + Caronamide	0.06 0.11	0.06 0.45	0.03 0.22	0.06 0.22
E. S.	M	Penicillin alone + Caronamide	<0.03 <0.03	<0.03 0.03	0.11 0.22	0.03 0.06
P. S.	M	Penicillin alone + Caronamide	<0.03 0.03	0.06 0.06	0.06 0.22	0.03 0.06
P. R.	M	Penicillin alone + Caronamide	0.03 0.06	0.06 <0.03	0.03 0.03	<0.03 <0.03

Caronamide (2 Gm. P.O.) at 6, 8, and 10 A.M., 12 N., and 2 P.M.

Penicillin (100,000 units P.O.) at 8 and 10 A.M., 12 N., and 2 P.M.

(Table I). Similar results were obtained with the twenty-four paired blood samples obtained two hours after each dose of penicillin (Table II). The plasma penicillin concentrations were the same with and without caronamide in five instances, lower with caronamide once, and higher with caronamide in eighteen tests. The increase was twofold in ten, fourfold in four, and eightfold or more in four. Tabulation of the results obtained both one and two hours after the administration of penicillin indicates that the concurrent administration of caronamide was associated with an enhancement of the plasma penicillin concentrations in 80 per cent of the paired blood samples; in 40 per cent this increase was fourfold or greater as compared with the controls. In terms of actual plasma penicillin concentrations, 62 per cent of the blood samples obtained while the subjects were receiving caronamide had a concentration of 0.1 unit or more of penicillin per milliliter of plasma. In contrast,

only 14 per cent of the samples obtained during the control period, on penicillin alone, had concentrations of 0.1 unit or more of penicillin per milliliter of plasma.

Five subjects received 2 Gm. of caronamide and 100,000 units of penicillin by mouth every three hours during the day. Blood samples were obtained three hours after each dose and immediately preceding the next dose. In three of these subjects, on another day, penicillin alone was given and bleedings obtained on the same schedule. Plasma concentrations of penicillin were low in all instances. Only two of nine samples taken while the subjects were receiving penicillin alone contained measurable amounts of penicillin (0.03 unit per milliliter). When the subjects received caronamide in addition, four of twelve samples contained 0.03 unit per milliliter of penicillin. The advantage of caronamide appeared to be negligible. It is evident, therefore, that while caronamide in the dosage used served effectively to elevate the plasma penicillin concentration for a period of two hours after the oral administration of 100,000 units of penicillin, the effect was dissipated within three hours.

*Penicillin Administered Intramuscularly:* Two subjects received intramuscular injections of 25,000 units of penicillin and two received 50,000 units of penicillin at two-hour intervals from 8 A.M. to 4 P.M. On the following day, the same dosage schedule of penicillin was resumed and, in addition, the subjects received 2 Gm. of caronamide by mouth every two hours, from 6 A.M. to 4 P.M. The plasma penicillin concentrations obtained one hour after each injection of penicillin are shown in Table III.

TABLE III. EFFECT OF CARONAMIDE ON PLASMA PENICILLIN CONCENTRATION  
(BLOOD SAMPLES ONE HOUR AFTER EACH DOSE)

SUBJECT	SEX	DRUG	PLASMA PENICILLIN CONCENTRATION (UNITS/ML.)				
			9 A.M.	11 A.M.	1 P.M.	3 P.M.	5 P.M.
Penicillin: 25,000 Units I.M. Every Two Hours							
S. L.	F	Penicillin alone	0.03	0.11	0.06	0.11	0.11
		+ Caronamide	0.22	0.22	0.45	0.45	0.11
C. S.	M	Penicillin alone	0.11	0.06	0.11	0.06	0.11
		+ Caronamide	0.11	0.11	0.22	0.22	0.22
Penicillin: 50,000 Units I.M. Every Two Hours							
C. Y.	F	Penicillin alone	---	0.22	0.22	0.22	0.11
		+ Caronamide	---	0.22	0.90	0.45	0.45
L. P.	F	Penicillin alone	---	0.45	0.11	0.22	0.11
		+ Caronamide	---	0.22	0.22	0.45	0.22

Caronamide (2 Gm. P.O.) at 6, 8, and 10 A.M. 12 N., 2 and 4 P.M.

Penicillin I.M. at 8 and 10 A.M. 12 N., and 2 and 4 P.M.

---, Blood sample not obtained.

In ten paired determinations in subjects on the 25,000 unit dosage schedule, the plasma penicillin concentrations were higher when caronamide was given in eight instances and the same in two instances. Seven of the ten blood specimens obtained when the subjects received caronamide had plasma concentrations of penicillin of 0.2 unit per milliliter or more. When the same subjects received penicillin alone, none of the ten specimens contained more than 0.1 unit of penicillin per milliliter of plasma.

In the subjects who received 50,000 units of penicillin, plasma penicillin concentrations were again higher (in six of eight paired specimens) when caronamide was administered concurrently. In general, the subjects who received caronamide with 25,000 units of penicillin had about the same plasma penicillin concentrations as those who received twice as much penicillin without caronamide.

Caronamide also effectively elevated the penicillin concentrations in the plasma of three other individuals who received 40,000 units of penicillin intramuscularly and 2 Gm. of caronamide by mouth at three-hour intervals during the day (Table VI). Blood samples were obtained one and one-half hours after each dose. Plasma penicillin concentrations were increased twofold or more in ten of twelve paired blood samples.

One of the subjects who had previously been given 50,000 units of penicillin was given two injections of 100,000 units of penicillin intramuscularly at 8 A.M. and 12 N. The following day, he was given the same amount of penicillin at the same hours, and, in addition, took 2 Gm. of caronamide at 6, 8, and 11 A.M. and 2 P.M. Plasma penicillin concentrations obtained one, three, and four hours after each dose of penicillin were identical on each day.\*

TABLE IV. EFFECT OF CARONAMIDE ON PLASMA PENICILLIN CONCENTRATION (SINGLE I.M. INJECTIONS OF PENICILLIN)

DOSE OF PENICILLIN	SUBJECT	SEX	WITHOUT CARONAMIDE				WITH CARONAMIDE			
			PLASMA PENICILLIN CONCENTRATION (UNITS/ML.)							
			HOURS AFTER PENICILLIN							
			1	2	3	4	1	2	3	4
25,000 units I.M.	L. W.	F	0.06	<0.03	<0.03	<0.03	0.06	0.03	0.03	<0.03
	E. A.	M	0.06	<0.03	<0.03	<0.03	0.11	0.03	<0.03	<0.03
	G. W.	M	0.03	0.03	0.03	<0.03	0.11	0.06	0.03	<0.03
50,000 units I.M.	H. R.	M	0.22	0.06	0.03	<0.03	0.11	0.06	0.03	0.03
	N. W.	F	0.11	0.03	<0.03	<0.03	0.22	0.06	0.03	0.03
	S. W.	F	0.22	0.06	<0.03	<0.03	0.22	0.11	0.06	0.03
100,000 units I.M.	H. R.	M	0.45	0.06	0.03	<0.03	0.22	0.06	0.06	<0.03
	B. H.	M	0.45	0.06	0.03	<0.03	0.45	0.22	0.11	0.06

Caronamide (2 or 3 Gm. P.O.) one hour before and at the time of administration of penicillin.

In eight subjects, plasma penicillin concentrations were determined at intervals following single intramuscular injections of penicillin. On the afternoon of the same day or the next day, the penicillin injections were repeated and, in addition, 2 or 3 Gm. of caronamide were given one hour before the penicillin injection and again at the time of the injection. The results which are recorded in Table IV indicate no significant effects of caronamide on the plasma penicillin concentrations. The discrepancy between these findings and those recorded in the foregoing may be due to the pharmacologic properties of caronamide. It would appear that the substance is either slowly absorbed or else requires a considerable period of time before it exerts its depressant effects on the renal excretory apparatus.

*Penicillin Administered in Peanut Oil and Beeswax:* The effect of caronamide on plasma penicillin concentrations eighteen and twenty-four hours after

\*Subsequent experience with other subjects has indicated that caronimide is effective in elevating plasma penicillin concentrations as much as fourfold, when doses of 200,000 units of penicillin are administered every three hours.

the injection of 600,000 units of penicillin in peanut oil and beeswax was determined in twelve patients with early syphilis.\* On the first day of the test, 600,000 units of penicillin (2 ml.) were administered intramuscularly in the buttock at 2 P.M.; at the same time, 1½ Gm. of caronamide were given by mouth and its administration continued thereafter every two hours until noon of the following day. Blood samples were obtained at 8 A.M. (eighteen hours) and at 2 P.M. (twenty-four hours) on the second day. Immediately after the second blood sample was obtained another injection of 600,000 units of penicillin in peanut oil and beeswax was given in the other buttock. No further

TABLE V. EFFECT OF CARONAMIDE ON PLASMA PENICILLIN CONCENTRATION  
(PENICILLIN IN PEANUT OIL AND BEESWAX: 600,000 UNITS I.M.)

SUBJECT	SEX	HOURS AFTER PENICILLIN	PLASMA PENICILLIN CONCENTRATION (UNITS/ML.)	
			PENICILLIN ALONE	PENICILLIN + CARONAMIDE
C. P.	M	18	0.03	0.22
		24	<0.03	0.45
D. H.	F	18	0.03	0.11
		24	0.06	0.11
A. E.	M	18	0.03	0.11
		24	<0.03	<0.03
C. B.	M	18	<0.03	0.11
		24	<0.03	0.45
E. P.	M	18	0.03	0.06
		24	<0.03	0.03
R. S.	F	18	<0.03	0.06
		24	<0.03	0.11
W. D.	M	18	<0.03	0.06
		24	<0.03	<0.03
S. G.	M	18	0.11	0.11
		24	0.06	0.11
L. M.	F	18	0.11	0.11
		24	<0.03	0.03
O. F.	M	18	0.03	0.03
		24	0.11	<0.03
M. C.	F	18	0.06	0.03
		24	0.03	0.22
E. B.	M	18	0.06	<0.03
		24	0.11	0.03

Caronamide (1½ Gm. P.O.) every two hours for twenty-four hours.

caronamide was given. Blood samples again were taken eighteen and twenty-four hours after the second injection of penicillin. The results are shown in Table V. In the majority of instances plasma penicillin concentrations were higher when caronamide was also given; however, the results were somewhat erratic and difficult to evaluate. Of twenty-four paired blood samples, the penicillin concentrations were higher when caronamide was given in fifteen, the same with and without caronamide in five, and lower with caronamide than without it in four instances. Less than a measurable amount of penicillin (<0.03 units per milliliter) was present in ten samples obtained in the absence of caronamide and in four samples in the presence of caronamide. Moreover,

\*These patients were studied by courtesy of Dr. A. G. Schoch, Dr. L. J. Alexander, and Dr. T. L. Shields, whose cooperation and assistance are gratefully acknowledged.

six of the twelve patients had plasma penicillin concentrations of 0.1 unit or more per milliliter while receiving caronamide, as compared with only two who obtained this concentration of penicillin in the plasma when not receiving caronamide.

*Effect of Caronamide on Plasma Streptomycin Concentrations.*—The possible value of caronamide in enhancing and prolonging streptomycin plasma concentrations was investigated in four subjects. Two subjects were given intramuscular injections of 0.5 Gm. of streptomycin, at 8 A.M. and 4 P.M., and caronamide was given by mouth every two hours from 6 A.M. to 10 P.M. (total dose, 18 Gm.). Blood samples were obtained two, six, and eight hours after each injection of streptomycin. On the following day the same schedule was followed except for the omission of caronamide. Within the limits of the method, the plasma concentrations of streptomycin were the same whether or not caronamide was given. Two other subjects received single injections of 0.5 Gm. of streptomycin intramuscularly. Blood samples were obtained at intervals for six hours afterward. On the following day, the streptomycin injections were repeated and 2.0 Gm. of caronamide were given by mouth at two-hour intervals for five doses beginning two hours before the streptomycin injection. In one subject, the concentration of streptomycin in the plasma was from one and one-half to two times higher when the caronamide was administered; in the other subject, however, the streptomycin concentrations for six hours after injection were the same with and without the concurrent administration of caronamide.

*Effect of Sodium Benzoate Alone and Together With Caronamide.*—

*Penicillin Administered Intramuscularly:* Three individuals received 40,000 units of penicillin intramuscularly every three hours from 8 A.M. to 5 P.M. on each of three consecutive days. On the first day of the test, 2 Gm. of caronamide were taken by mouth at 6, 8, and 11 A.M., and 2 and 5 P.M. On the third day of the test, 2 Gm. of sodium benzoate were taken by mouth at similar hours. Blood samples were obtained one and one-half hours after each injection of penicillin. The plasma penicillin concentrations are recorded in Table VI.

TABLE VI. EFFECT OF CARONAMIDE AND SODIUM BENZOATE ON PLASMA PENICILLIN CONCENTRATION (PENICILLIN: 40,000 UNITS I.M. EVERY THREE HOURS; BLOOD SAMPLES ONE AND ONE-HALF HOURS AFTER EACH DOSE)

SUBJECT	SEX	DRUG	PLASMA PENICILLIN CONCENTRATION (UNITS/ML.)			
			9:30 A.M.	12:30 P.M.	3:30 P.M.	6:30 P.M.
E. C.	F	Penicillin alone	0.11	0.06	0.06	0.03
		+ Caronamide	0.22	0.06	0.11	0.11
		+ Sodium benzoate	0.22	0.11	0.06	0.06
M. H.	F	Penicillin alone	0.11	0.06	0.06	0.06
		+ Caronamide	0.22	0.11	0.22	0.45
		+ Sodium benzoate	0.22	0.45	0.45	0.06
J. D.	F	Penicillin alone	0.22	0.06	0.06	0.06
		+ Caronamide	0.22	0.11	0.22	0.22
		+ Sodium benzoate	0.06	0.11	0.22	0.11

Caronamide and sodium benzoate (2 Gm. P.O.) at 6, 8, and 11 A.M., 2 and 5 P.M.  
Penicillin (40,000 units I.M.) at 8 and 11 A.M., and 2 and 5 P.M.



Both caronamide and sodium benzoate effectively increased plasma penicillin concentrations. The two drugs appeared to be equally effective since the penicillin concentrations were the same with caronamide as with sodium benzoate four times, higher with caronamide than with sodium benzoate five times, and lower with caronamide than with sodium benzoate three times.

*Penicillin Administered by Mouth:* In three subjects who received penicillin by mouth, a comparison was made of the value of caronamide and sodium benzoate individually and together in increasing the penicillin concentration of the plasma. Penicillin, 100,000 units by mouth, was administered every two hours from 8 A.M. to 2 P.M. on each of four consecutive days. On the first day of the test, 2 Gm. of caronamide were also taken by mouth at 6, 8, and 10 A.M., 12 N., and 2 P.M. On the second day, 2 Gm. of sodium benzoate were taken at the same hours in place of the caronamide. On the third day, neither caronamide nor sodium benzoate was administered with the penicillin. On the fourth day both caronamide and sodium benzoate, 2 Gm. of each, were taken at two-hour intervals from 6 A.M. to 2 P.M. Blood samples were secured two hours after each dose of penicillin on each day of the test. The resulting plasma penicillin concentrations are recorded in Table VII (part of the data is also included in Table II).

TABLE VII. EFFECT OF CARONAMIDE AND SODIUM BENZOATE, INDIVIDUALLY AND IN COMBINATION, ON PLASMA PENICILLIN CONCENTRATION  
(PENICILLIN: 100,000 UNITS BY MOUTH EVERY TWO HOURS;  
BLOOD SAMPLES TWO HOURS AFTER EACH DOSE)

SUBJECT	SEX	DRUG	PLASMA PENICILLIN CONCENTRATION (UNITS/ML.)			
			10 A.M.	12 N.	2 P.M.	4 P.M.
E. S.	M	Penicillin alone	<0.03	<0.03	0.11	0.03
		+ Caronamide	<0.03	0.03	0.22	0.06
		+ Sodium benzoate	0.03	0.06	0.06	0.11
		+ Combination	0.06	0.22	0.22	0.22
P. S.	M	Penicillin alone	<0.03	0.06	0.06	0.03
		+ Caronamide	0.03	0.06	0.22	0.06
		+ Sodium benzoate	0.11	0.06	0.11	0.11
		+ Combination	0.06	0.22	0.90	0.45
P. R.	M	Penicillin alone	0.03	0.06	0.03	<0.03
		+ Caronamide	0.06	<0.03	0.03	<0.03
		+ Sodium benzoate	0.06	0.06	0.06	0.03
		+ Combination	0.22	0.06	0.11	0.06

Caronamide and sodium benzoate, each (2 Gm. P.O.) at 6, 8, and 10 A.M., 12 N., and 2 P.M.  
Penicillin (100,000 units P.O.) at 8 and 10 A.M., 12 N., and 2 P.M.

In these subjects, caronamide alone was relatively ineffective in raising the penicillin concentration. A twofold or greater increase (as compared with the controls) was recorded in seven of twelve blood samples, but in only one specimen was there a fourfold increase. Sodium benzoate was more effective than caronamide. Three-fourths of the blood samples obtained when sodium benzoate was taken contained more penicillin than the controls; but here, too, in the majority the increase was not greater than twofold. In contrast, the combination of both caronamide and sodium benzoate resulted in a marked enhancement of plasma penicillin concentrations and yielded better results than either drug alone. A fourfold or greater increase in the penicillin con-

centration was obtained in ten of the twelve blood samples, as compared with the controls. Two-thirds of the blood samples obtained on this regimen contained 0.1 unit of penicillin per milliliter of plasma, and none contained less than 0.06 unit.

It was previously demonstrated that measurable amounts of penicillin did not persist in the blood for three hours after the oral administration of 100,000 units of penicillin, taken alone or with caronamide. It was, therefore, of interest to determine the duration of the effect of both caronamide and sodium benzoate. Two subjects took 100,000 units of penicillin and 2 Gm. each of caronamide and sodium benzoate by mouth every three hours during the day. Blood samples were obtained three hours after each dose of penicillin. In five of six blood samples obtained, measurable concentrations of penicillin (0.03 unit or more) were present.

*Toxicity of Caronamide and Sodium Benzoate:* Approximately fifty adult subjects took caronamide by mouth. The majority received the drug for one day or less; a few took it for as long as three days. The maximum total daily dosage was 18 Gm.; the majority took 10 to 12 Gm. within a twelve-hour period. Gastrointestinal intolerance was the only evidence of toxicity noted. Two subjects complained of nausea and mild diarrhea, the latter persisting for two days. These symptoms began, however, the day after the administration of caronamide and at a time when the subjects were continuing to take penicillin by mouth. The intolerance cannot clearly be ascribed, therefore, to caronamide. Two other subjects complained of nausea and diarrhea for one day coincident with the ingestion of penicillin, caronamide, and sodium benzoate. Again it is impossible to decide which of the three drugs or whether the combination of them was responsible for the gastrointestinal disturbance. Since this reaction occurred in two of the five subjects who received all three drugs at the time, it is likely that a considerable percentage of individuals placed on this regimen will be unable to tolerate it.

#### DISCUSSION

The observation that a combination of caronamide and sodium benzoate resulted in higher penicillin blood concentrations than were attained by the use of each drug alone is of particular interest with regard to the mode of action of these agents. Two possible explanations are suggested: (1) that the dose of each drug was inadequate for maximal effect and (2) that caronamide and sodium benzoate act by different mechanisms. Since a rather fixed dosage schedule of caronamide was used in these studies, the validity of the first explanation cannot be established. It may be noted, however, that the total dose of caronamide usually given within four or six hours was close to that required to produce complete suppression of tubular excretion of penicillin in dogs.<sup>5</sup> The assumption has been made that benzoic acid and sodium benzoate act in a manner similar to diodrast<sup>7</sup> and p-aminohippurate,<sup>8</sup> competing with penicillin for a common renal tubular excretory mechanism. The evidence of Beyer bearing on the mode of action of caronamide has been referred to previously.<sup>4</sup> Recently, evidence has been presented that sodium benzoate has an antipeni-

cillinase effect.<sup>13</sup> It has been suggested that this property, rather than a blocking action on the renal tubules, is responsible for the observed action of sodium benzoate. If such were the case, the apparent synergistic effect of caronamide and sodium benzoate could be explained as the summation of two different mechanisms, each of which helps to maintain the penicillin concentration in the blood. In vitro we have noted that caronamide also exhibits an antipenicillinase effect but do not believe that this action offers an explanation for its behavior in the body, especially since normal pooled plasma also exhibits an antipenicillinase effect which is greater than that of caronamide.

The present studies appear to be sufficiently detailed to indicate the probable value of caronamide as an adjunct to penicillin therapy. It is clear that caronamide is effective in elevating the penicillin concentration in the blood; however, for this purpose, its effectiveness appears to be no greater than that exhibited by sodium benzoate. The choice of either drug would seem therefore to depend in part on the respective toxicity of the two agents. Sodium benzoate is regarded as an innocuous substance,<sup>14</sup> its principal disadvantage being its rather frequent nauseant action. Enough experience has not as yet accumulated to assess the incidence of toxic reactions or idiosyncrasies to caronamide. Until such experience is gained under controlled conditions of observation, its routine use in preference to sodium benzoate is not recommended.

When penicillin was given intramuscularly in aqueous solution, the concurrent administration of caronamide or sodium benzoate resulted in a twofold or greater increase in the concentration of penicillin in the plasma in most instances. However, no studies were made as to the total duration of this effect of caronamide. Essentially the same blood concentrations of penicillin were attained with the injection of 25,000 units of penicillin plus caronamide as resulted from the injection of 50,000 units of penicillin without an adjuvant. Such blood concentrations of penicillin are probably entirely adequate for the control of infections due to organisms ordinarily responsive to penicillin therapy, except for those due to unusually resistant strains. If toxicity and intolerance do not prove to be limiting factors in the use of caronamide, this drug plus 25,000 units of penicillin, intramuscularly, every two hours, will probably result in the maintenance of adequate blood penicillin concentrations. Such a regimen, however, while saving of penicillin, does not obviate the need for repeated injections, which is one of the chief disadvantages of penicillin treatment. From a practical viewpoint, therefore, the chief interest in such substances as caronamide and sodium benzoate lies in their possible value in enhancing the effectiveness of penicillin when given by mouth or in single daily injections in peanut oil and beeswax.

Although it is impossible to define an adequate level of penicillin, experience with in vitro tests indicates that penicillin concentrations of 0.03 to 0.06 unit per milliliter are generally adequate for control of infection with the majority of strains of pneumococcus, hemolytic streptococcus of group A, gonococcus, and *Streptococcus viridans*. From two to eight times as much penicillin may be needed for the control of infections due to some strains of these organisms as well as to many strains of staphylococcus and meningococcus.<sup>15</sup> In

the present study, when 100,000 units of penicillin were taken orally every two hours, adequate plasma penicillin concentrations were not achieved in a large proportion of the blood samples. Two-thirds of the blood specimens obtained one hour after this oral dose of penicillin, and one-half of the specimens obtained two hours after this dose, contained 0.03 unit or less per milliliter.

Caronamide was of definite value in enhancing the penicillin concentration in the plasma when penicillin was given by mouth. In 80 per cent of the blood samples obtained one and two hours after the ingestion of 100,000 units of penicillin, the administration of caronamide was associated with a twofold or greater increase in the plasma penicillin concentration: in 40 per cent the increase was fourfold or greater over the corresponding control samples. When the subjects took caronamide, 70 per cent of the blood specimens contained 0.1 unit or more per milliliter one hour after the dose of penicillin, and 54 per cent of the samples still contained 0.1 unit or more per milliliter two hours after the dose of penicillin. Unfortunately, caronamide did not maintain adequate penicillin concentrations for three hours. The effect of sodium benzoate was essentially the same as that of caronamide for two hours after the oral dose of penicillin. The duration of effect of this drug was not measured beyond two hours, but the data strongly suggested that sodium benzoate likewise does not maintain effective plasma penicillin concentrations for three hours after a dose of 100,000 units of penicillin orally. The combination of sodium benzoate and caronamide together with 100,000 units of penicillin orally, every three hours, resulted in optimal plasma concentrations for two hours and adequate concentrations of penicillin for three hours. Although this regimen appears to yield good results, it seems likely that a considerable number of patients will be unable to tolerate it.

While the foregoing data suggest the value of caronamide or sodium benzoate as adjuvants for the successful use of orally administered penicillin, they omit consideration of a serious limitation: namely, the differences in response which individual subjects exhibited. When oral penicillin was given alone, variations in the plasma concentrations attained were evident not only in different subjects, but also in the same subjects from hour to hour. The time of ingestion of meals and the fluid intake have an important influence on blood concentrations of penicillin,<sup>1, 16</sup> and such influences were undoubtedly responsible for some of the variations observed. When caronamide was taken, however, the individual variations in plasma penicillin concentrations were greatly accentuated. For example, Subject P. R. (Tables I, II, and VII) consistently exhibited a poor response to caronamide and to sodium benzoate; Subject N. P. (Table I), on the contrary, had an unusually good effect from the administration of caronamide. Such unpredictable individual variations, while recognized in the pharmacology of all drugs, have been particularly noted with the oral administration of penicillin.<sup>3</sup> They render uncertain any attempt to define minimal effective dosage schedules for penicillin, which can be used routinely without the necessity of measuring the blood concentrations.

In keeping with the findings of others,<sup>17</sup> the results in the present study indicate that only a minority of patients maintain an effective concentration

of penicillin in the blood twenty-four hours after the intramuscular injection of 600,000 units of penicillin in peanut oil and beeswax. Less than 0.03 unit of penicillin per milliliter of plasma was present at twenty-four hours in seven of twelve patients, and only two had concentrations of 0.1 unit or more. It is, therefore, of interest that when caronamide was administered throughout the twenty-four hours following the injection of penicillin in wax and oil, only three of twelve had plasma penicillin concentrations of less than 0.03 unit per milliliter, and six had 0.1 unit or more per milliliter of plasma. The inconsistencies in the results, however, are such that a definite opinion as to the value of caronamide cannot be expressed. Individual variations are undoubtedly of importance when penicillin is administered by this route as well as by mouth. and, in addition, such factors peculiar to this route of administration as the accuracy of measurement of the dosage, variations in the site of deposition of the penicillin in the tissues, and amount of muscular activity, influence the resulting plasma penicillin concentrations.

The foregoing discussion implies a field of usefulness for caronamide and sodium benzoate which practical considerations may negate. Despite their effect, it is believed that these drugs will prove to be of only limited value when penicillin is given by intermittent intramuscular injection. The saving in the cost of penicillin may be more than offset by the inconvenience and potential toxicity of additional medications.\* A similar conclusion probably also will soon apply to penicillin in peanut oil and beeswax. Larger doses in more convenient preparations will obviate the need for adjuvants such as these.

In the field of oral penicillin therapy these substances may have greater usefulness. In terms of the plasma penicillin concentrations attained, oral therapy is not only inefficient and costly, but also unreliable. The addition of caronamide or sodium benzoate, while not permitting any reduction in the dosage of oral penicillin, at least appears to assure more satisfactory blood concentrations than can be obtained without them.

#### SUMMARY AND CONCLUSIONS

1. The effectiveness of caronamide (4'-carboxy-phenylmethanesulfonanilide) in enhancing the plasma penicillin concentration was studied in thirty-five adult subjects. This effect was compared with that of sodium benzoate.

2. The only toxic effects of caronamide encountered were nausea and mild diarrhea in a few individuals.

3. The administration of 100,000 units of penicillin by mouth resulted in low plasma concentrations one and two hours after each dose. The concurrent administration of caronamide resulted in a twofold or greater increase in plasma penicillin concentrations in 80 per cent of the blood samples, and a fourfold or greater increase in 40 per cent of the samples. This enhancement of the plasma penicillin concentration by caronamide was maintained for two hours but not for three hours after each dose of penicillin.

\*Experience acquired since the completion of this manuscript indicates that in situations in which more than a million units of penicillin per day are required, the concurrent administration of caronamide may be of considerable value. Increases of twofold or greater in plasma penicillin levels may be obtained, and result in considerable saving in the amounts of penicillin required.

4. In subjects who received 25,000 and 50,000 units of penicillin intramuscularly, the concurrent oral administration of caronamide likewise resulted in two- to eightfold increases in plasma penicillin concentrations in most instances. When penicillin was injected in peanut oil and beeswax the effects of caronamide were variable, but in the majority of instances plasma penicillin concentrations were increased.

5. Caronamide had no effect on the plasma concentration of streptomycin when the latter substance was administered in multiple intramuscular injections.

6. In general, sodium benzoate was as effective as caronamide in increasing plasma penicillin concentrations when penicillin was given by mouth or by intramuscular injection. The ingestion of both sodium benzoate and caronamide by subjects receiving penicillin by mouth resulted in a greater enhancement of plasma penicillin concentrations than was effected by either of these agents alone.

Technical assistance was given in these studies by Anna Maxell.

# REFERENCES

1. Bronfenbrenner, J., and Favour, C. B.: Increasing and Prolonging Blood Penicillin Concentrations Following Intramuscular Administration, *Science* 101: 673-674, 1945.
2. (a) Bohls, S. W., and Cook, E. B. M.: The Use of Aluminum-Penicillin Mixtures in Maintenance of Blood Levels of Penicillin. II. Combination of Delayed Absorption by Use of Aluminum-Penicillin and Renal Blockage With Benzoic Acid, *Texas State J. Med.* 41: 342-344, 1945.  
(b) Bohls, S. W., Cook, E. B. M., and Potter, R. T.: Oral and Parenteral Use of Aluminum-Penicillin Mixtures in the Treatment of Gonorrhea, *J. Ven. Dis. Inform.* 27: 69-74, 1946.
3. Spaulding, E. H., Bondi, A., and Early, E.: The Effect of Combining Sodium Benzoate With Oral Penicillins, *Science* 105: 210-211, 1947.
4. Beyer, K. H.: New Concept of Competitive Inhibition of the Renal Tubular Excretion of Penicillin, *Science* 105: 94-95, 1947.
5. Beyer, K. H., Miller, A. K., Russo, H. F., Patch, E. A., and Verwey, W. F.: The Inhibitory Effect of Caronamide on the Renal Elimination of Penicillin. Unpublished.
6. Beyer, K. H., Russo, H. F., Patch, E. A., and Tillson, E. A.: Caronamide: Its Pharmacological Properties. Unpublished.
7. Rammelkamp, C. H., and Bradley, S. E.: Excretion of Penicillin in Man, *Proc. Soc. Exper. Biol. & Med.* 53: 30-32, 1943.
8. Beyer, K. H., Peters, L., Woodward, R., and Verwey, W. F.: Enhancement of Physiological Economy of Penicillin in Dogs by Simultaneous Administration of Para-Aminohippuric Acid, *J. Pharmacol. & Exper. Therap.* 82: 310-323, 1944.
9. Beyer, K. H., McKinney, S. E., Tillson, E. A., and Green, C. W.: Caronamide: Its Toxicologic Effects. Unpublished.
10. Rapoport, M., Beyer, K. H., Corneal, M. B., and Verwey, W. F.: A Clinical Evaluation of the Toxicity and Efficacy of Caronamide for Competitive Inhibition of Penicillin Excretion. Unpublished.
11. Crosson, J. W., Boger, W. P., Shaw, C. C., and Miller, A. K.: Enhancement of Penicillin Plasma Concentration With Caronamide. Unpublished.
12. Rammelkamp, C. H.: A Method of Determining the Concentration of Penicillin in Body Fluids and Exudates, *Proc. Soc. Exper. Biol. & Med.* 51: 95-97, 1942.
13. Reid, R. D., Felton, L. C., and Pittroff, M. A.: Prolongation of Penicillin Activity With Penicillinase-Inhibiting Compounds, *Proc. Soc. Exper. Biol. & Med.* 63: 438-443, 1946.
14. Quick, A. J.: Letter to the Editor, *J. A. M. A.* 124: 1219-1220, 1944.
15. Meads, M., Ory, E. M., Wilcox, C., and Finland, M.: Penicillin Sensitivity of Strains of Six Common Pathogens and of *Hemophilus Hemolyticus*, *J. LAB. & CLIN. MED.* 30: 725-730, 1945.
16. Finland, M., Meads, M., and Ory, E. M.: Oral Penicillin, *J. A. M. A.* 129: 315-320, 1945.
17. Hirsh, H. L., Dowling, H. F., Vivino, J. J., and Rotman-Kavka, G.: Penicillin in Beeswax and Peanut Oil, a New Preparation Which Is Fluid at Room Temperature: Absorption and Therapeutic Use, *J. LAB. & CLIN. MED.* 32: 24-41, 1947.

# A WATER-SOLUBLE PREPARATION FOR PROLONGING EFFECTIVE PENICILLIN LEVELS IN BODY FLUIDS

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THE exigencies of routine clinical practice frequently call for the use of a single daily dose injectible preparation of penicillin that can be relied upon to obtain and maintain effective therapeutic levels of the antibiotic in body fluids. The preparation, ideally, must be stable, water soluble, nontoxic, and nonirritant, and it must be capable of easy and safe administration.

To meet these basic criteria, the following formula\* has been developed and employed successfully:

Crystalline sodium salt of penicillin G (300,000 Oxford units)	180.0 mg.
Ephedrine sulfate	25.0 mg.
Epinephrine dihydrochloride	1.0 mg.
Eucupine dihydrochloride	1.0 mg.
Gelatin-dextrose mixture (dried weight)	800.0 to 1,200.0 mg.

When sterilized, dehydrated, and bottled, the preparation appears as a homogeneous powder. On the addition of 2 ml. of distilled water, the powder dissolves readily at room temperature and with great facility if the container is agitated or gently heated by immersion in hot tap water. The resultant amber-colored, water-clear solution may then be drawn up into a 5 or 10 ml. syringe through an 18- or 19-gauge needle and injected without undue haste, subcutaneously or intramuscularly, using a 20-gauge needle. A delay in injection of more than thirty minutes at room temperature may solidify the gel which can then again be fluidified by gentle heat as indicated.

## RATIONALE

The vehicle, as described, is composed of penicillin, vasoconstrictors, a local anesthetic, and the gelatin-dextrose mixture. The final pH of the dehydrated preparation, as bottled, is 6.0.

Crystalline sodium penicillin G, 300,000 Oxford units, is the conventional dose for repository injection. It is noteworthy that special penicillins, such as the calcium salt, are not essential for this preparation. If it is desirable to augment the standard dosage, it can be done by merely incorporating additional penicillin in the 2 ml. sterile distilled water which is used to reconstitute the preparation.

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The vasoconstrictors are included for additive prolonging effects. Following the injection, because of the vasoconstrictor drugs, the patient may occasionally complain of transitory palpitation and nervousness. No objective vasoconstrictor effects have been observed in any of the subjects.

The eucupine dihydrochloride serves as a local anesthetic. The hydrogen-ion concentration of 6.0 is optimal. With this pH the penicillin remains stable at room temperature for long periods of time. Furthermore, there is no deterioration of the vasoconstrictor drugs nor is there precipitation of the eucupine dihydrochloride.

In the course of our studies with heparin, the Pitkin menstruum was adopted as the vehicle in order to accomplish a slower and more equable absorption of the drug and to supplant the cumbersome technique of intravenous administration.<sup>1, 2</sup> The Pitkin menstruum was devised for regulating and retarding the rate of release of water-soluble drugs injected subcutaneously or intramuscularly. The clinical department of heparin in the Pitkin menstruum has been reported on several occasions.<sup>3-5</sup>

The possibility of utilizing the Pitkin menstruum for penicillin was explored with only partially successful results because both the water content and the low pH induced by the acetic acid in the menstruum were deleterious to penicillin.

These shortcomings were overcome by (1) the selection of gelatins which are more compatible with penicillin and more physiologically acceptable by body tissues, (2) elimination of acetic acid, (3) adjustment of pH, and (4) dehydration of the preparation.

The selection of a satisfactory gelatin\* is predicated on several factors. While gelatins are nonallergenic and therefore may be employed in preparing vehicles for intramuscular injections, they differ considerably in their chemical and physical properties, depending upon the source material and method of extraction.

The gelatin of choice must have a suitable molecular weight and jelly strength. To be of practical value this gelatin must lend itself readily to dehydration and reconstitution in aqueous solution at room temperature.

#### TECHNIC OF ADMINISTRATION

The steps in the administration of the preparation have been previously indicated but may be summarized as follows:

1. Warm the bottle by immersion in hot water and/or by agitation.
2. Expose the rubber diaphragm and sterilize with alcohol; inject 2 ml. of sterile distilled water.
3. Reimmerse the bottle in warm water in a basin or under the tap.
4. With an 18- or 19-gauge needle withdraw the solution into a 5 or 10 ml. sterile syringe.
5. Substitute a 20-gauge needle and inject the gel subcutaneously or intramuscularly, first withdrawing the piston slightly to make sure that a vein has not inadvertently been entered.

\*We wish to express our appreciation to Dr. Thomas E. Downey, of the Kind-Knox Gelatin Co., Camden, N. J., for his interest and cooperation in this phase of our problem.



## RESULTS

The clinical department of the various dehydrated penicillin and gelatin-dextrose preparations has been observed in over 300 subjects. The series included normal controls and ambulatory and bed patients with and without infections. These clinical observations were for the purpose of establishing the composition of the optimum preparation, having in mind its anti-infective properties, its effectiveness in prolonging penicillin levels in body fluids, the absence of toxicity, freedom from local reactions, and ease of administration. Stability studies on the preparation were also carried out with bottles that were stored at room temperature for more than a year.

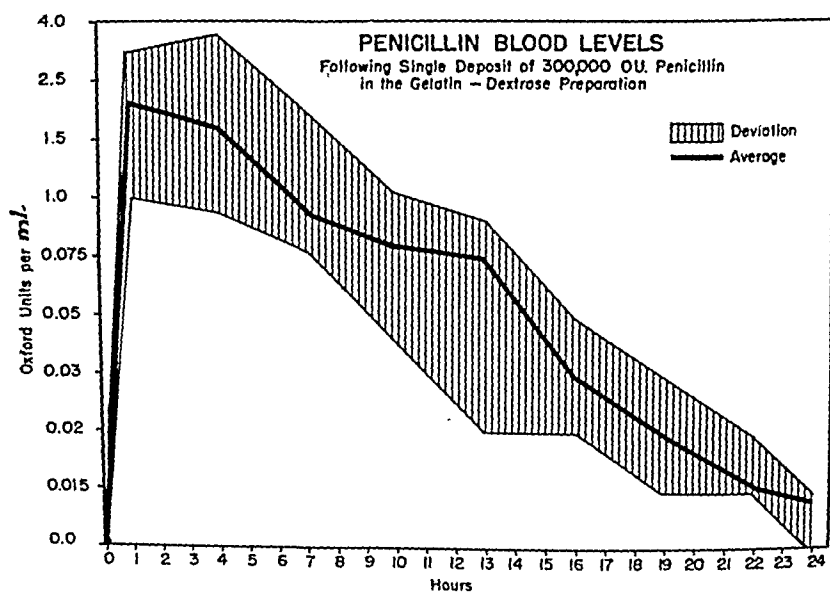


Fig. 1.

Analysis of data (Table I) and a composite graph (Fig. 1) of blood levels\* of twenty-four consecutive patients disclose measurable amounts of penicillin up to twenty-four hours following a single dose of 300,000 Oxford units in the gelatin-dextrose preparation. The pattern of blood levels in this group of twenty-four patients is characteristic for all subjects receiving the optimum preparation. The average blood penicillin levels are at peak during the initial eight to thirteen hours (0.08 to 1.91 Oxford units per milliliter) and then taper off to a level (0.015 Oxford units per milliliter) which is still bacteriostatic for many of the ordinary penicillin-inhibitable infecting organisms. These include most strains of gonococci, pneumococci, hemolytic streptococci, and staphylococci. In no instance has the preparation of choice been toxic or productive of local reactions.

\*The penicillin assay method used was that described by Rosenblatt, Altire-Werber, Kashdan and Loewe,<sup>6</sup> which employs *Streptococcus pyogenes* C203 as test organism. In a comparative study this system was found by Dolkart, Dey, and Schwemlein<sup>7</sup> to yield generally and, at times, appreciably lower values than the Food and Drug Administration assay procedure described by Randall, Price, and Welch,<sup>8</sup> which uses *Bacillus subtilis* NRRL B-558 as the test organism.

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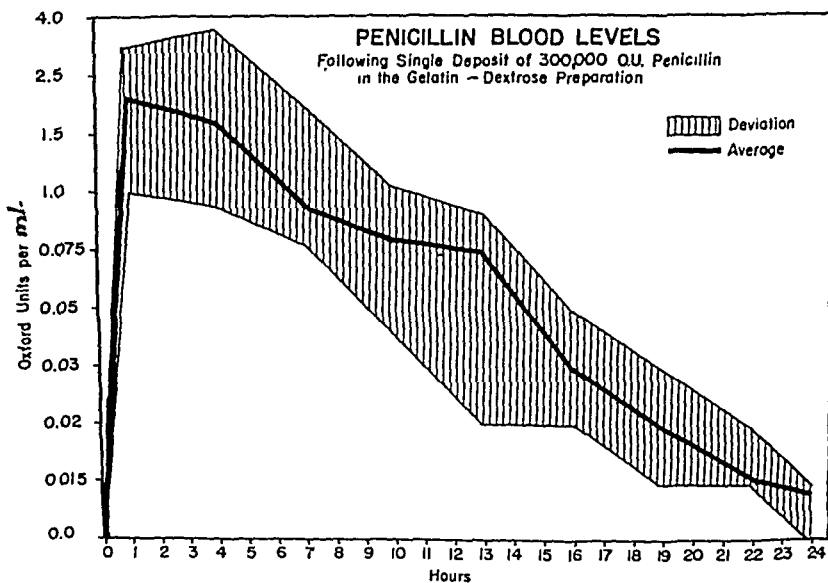


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TABLE I. ANALYSIS OF BLOOD PENICILLIN LEVELS IN A SERIES OF TWENTY-FOUR PATIENTS INJECTED (SUBCUTANEOUSLY OR INTRAMUSCULARLY) WITH 300,000 UNITS OF PENICILLIN IN THE GELATIN-DEXTROSE PREPARATION

NUMBER OF PATIENTS TESTED	1 HR.	4 HR.	7 HR.	10 HR.	13 HR.	16 HR.	19 HR.	22 HR.	24 HR.
	24	24	24	24	24	23	22	16	6
Number of patients with a level of 0.03 units per milliliter or more	24	24	24	24	23	15	4	0	0
Per cent	100	100	100	100	95	65	18	0	0
Average level (from all deter- minations) units per milliliter	1.91	1.62	0.89	0.35	0.09	0.03	0.03	0	0
Number of patients with a level of 0.015 units per milliliter or more	24	24	24	24	24	23	22	16	3
Per cent	100	100	100	100	100	100	100	100	500.0
Average level (from all deter- minations) units per milliliter	1.91	1.62	0.89	0.35	0.08	0.03	0.02	0.016	50

The therapeutic application of this preparation has been studied in various diseases such as gonorrhea, pneumococcus pneumonia, streptococcus sore throat, and furunculosis. The results of these studies will be the subject of subsequent reports.

#### DISCUSSIONS

Ever since penicillin became available for clinical use, attempts have been made to devise methods for administering this agent which would overcome its inordinately rapid loss through the kidneys. Although this problem has engaged the attention of numerous investigators, recourse, for the most part, must still be made to conventional, fractional intramuscular injections or continuous venoclysis.

Even with two-hour intermittent, intramuscular injections, there is a fraction of the treatment period wherein there is little or no assayable penicillin in the blood. About 60 per cent of the parenterally administered penicillin is excreted in the urine, mostly within one hour after intramuscular injection of the aqueous solution. As a result, in some infections treatment failure is almost inevitable. This is especially true of infections caused by the more resistant types of organisms. Furthermore, in long-continued infections repeated intramuscular injections become irksome and distressing. Continuous intravenous administration is resorted to in these circumstances, usually with gratifying results. The continuous venoclysis route is still the method of choice, however, where uninterrupted massive dosages are required over extended periods of time, as in the treatment of subacute bacterial endocarditis.<sup>9, 10</sup> It may even be necessary to incorporate in the treatment program some potentiating agent such as sodium para-aminohippurate in order to achieve requisite blood penicillin assays.<sup>10-13</sup>

Penicillin has been emulsified in beeswax and peanut oil<sup>14</sup> and in oil and water mixtures<sup>15</sup> as a means of retarding absorption and prolonging the action

of penicillin and thus obviating frequent repeated intramuscular injections necessary with aqueous solutions. While the penicillin assays in body fluids of patients so treated reveal adequate levels for acute infections, the continued use of these foreign insoluble vehicles has certain disadvantages. Local reactions are common,<sup>16</sup> and foreign body tissue responses have been reported following these repositories.<sup>17</sup> Additional drawbacks of the oil and beeswax preparations are lack of stability at room temperature and technical difficulties of administration. For these reasons a water-soluble, wholly absorbable, stable, nontoxic vehicle for penicillin is preferable.

#### SUMMARY AND CONCLUSIONS

A new penicillin preparation for prolonging effective levels in body fluids has been described. It is water-soluble, nontoxic, free of local reactions, and easy to administer. It maintains measurable levels in the blood for periods up to twenty-four hours or longer and has retained its stability at room temperature for more than a year.

#### REFERENCES

1. Loewe, L., Rosenblatt, P., and Lederer, M.: A New Method of Administering Heparin, *Proc. Soc. Exper. Biol. & Med.* 30: 53, 1942.
2. Loewe, L., and Rosenblatt, P.: A New Practical Method of Administering Heparin: Preliminary Report, *Am. J. M. Sc.* 208: 54, 1944.
3. Loewe, L., Rosenblatt, P., and Hirsch, E.: Venous Thromboembolic Disease, *J. A. M. A.* 130: 386, 1946.
4. Loewe, L., and Hirsch, E.: Heparin in the Treatment of Thromboembolic Disease, *J. A. M. A.* 133: 1263, 1947.
5. Evans, J. A., and Boller, R. J.: The Subcutaneous Use of Heparin in Anticoagulation Therapy, *J. A. M. A.* 131: 879, 1946.
6. Rosenblatt, P., Altire-Werber, E., Kashdan, F., and Loewe, L.: A Method for the Determination of Penicillin Levels in Body Fluids, *J. Bact.* 48: 599, 1944.
7. Dolkart, R. E., Dey, F. L., and Schwemlein, G. X.: Penicillin Assay Techniques: A Comparative Study, *J. Bact.* 53: 17, 1947.
8. Randall, W. A., Price, C. W., and Welch, H.: The Estimation of Penicillin in Body Fluids, *Science* 101: 365, 1945.
9. Loewe, L.: The Combined Use of Penicillin and Heparin in the Treatment of Subacute Bacterial Endocarditis, *Canad. M. A. J.* 52: 1, 1945.
10. Loewe, L., Rosenblatt, P., Russell, M., and Altire-Werber, E.: The Superiority of the Continuous Intravenous Drip for the Maintenance of Effectual Serum Levels of Penicillin: Comparative Studies With Particular Reference to Fractional and Continuous Intramuscular Administration, *J. Lab. & Clin. Med.* 30: 730, 1945.
11. Beyer, K. H., Flippin, H., Verwey, W. F., and Woodward, R.: The Effect of Para-Aminohippuric Acid on Plasma Concentration of Penicillin in Man, *J. A. M. A.* 126: 1007, 1944.
12. Loewe, L., Rosenblatt, P., Altire-Werber, E., and Kozak, M.: The Prolonging Action of Penicillin by Para-Aminohippuric Acid, *Proc. Soc. Exper. Biol. & Med.* 58: 298, 1945.
13. Loewe, L., Rosenblatt, P., and Altire-Werber, E.: A Refractory Case of Subacute Bacterial Endocarditis Due to *Veillonella Gazogenes* Clinically Arrested by a Combination of Penicillin, Sodium Para-Aminohippurate and Heparin, *Am. Heart J.* 32: 327, 1946.
14. Romansky, M. J., and Rittman, G. E.: Penicillin: Prolonged Action in Beeswax-Peanut Oil Mixture; Single Injection Treatment of Gonorrhea, *Bull. U. S. Army M. Dept.* (No. 81), pp. 43-49, 1944; Abstr., *Method of Prolonging Action of Penicillin*, *Science* 100: 196, 1944.
15. Freund, J., and Thomson, K. J.: Simple, Rapid Technic of Preparing Water-in-Oil Emulsions of Penicillin, *Drugs and Biologics*, *Science* 101: 468, 1945.
16. Kirby, W. M. M., Leifer, W., Martin, S. P., and \_\_\_\_\_: Intramuscular and Subcutaneous Administration of Penicillin, *J. A. M. A.* 129: 940, 1945.
17. Romansky, M. J.: The Current Status of Calcium Penicillin in Beeswax and Peanut Oil, *Am. J. Med.* 1: 395, 1946.

# USE OF MODIFIED DUBOS AND DAVIS MEDIUM FOR DEMONSTRATION OF ANTIBIOTIC ACTIVITY OF SUBTILIN AGAINST MYCOBACTERIUM TUBERCULOSIS

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ATTEMPTS to demonstrate the antibiotic activity of subtilin in vitro against *Mycobacterium tuberculosis* by means of the classical Long's synthetic medium have yielded inconsistent results.<sup>1</sup> With any antibiotic, such as subtilin, which has been shown to lose 68 per cent of its activity when held at 37° C., pH 7.3, for six days,<sup>2</sup> a single, initial application has proved to be inadequate.<sup>3</sup> It is obvious that the results obtained at the end of three weeks' exposure (the usual growth period) do not represent initial activity. In addition, the classical method, which consists of floating the inoculum, does not permit intimate contact between the antibiotic and all mycobacteria present in the culture. In fact, only those organisms present in the base and periphery of the inoculum seem to be affected.

A recently developed liquid medium which appears to circumvent these difficulties is that of Dubos and Davis,<sup>4</sup> who made use of a synthetic wetting agent under the trade name of "Tween 80" to facilitate submerged growth, and a purified albumin (Cohn's Fraction V) to increase the growth rate of mycobacteria. In the present communication we wish to report our experience with the use of this medium to demonstrate the antibiotic activity of subtilin against a virulent strain of *Myco. tuberculosis*.

## MATERIALS AND METHODS

*Myco. tuberculosis*† was the organism employed. Two lots of subtilin, one of which was more toxic for mice than the second, were used. The composition of the slightly modified Dubos and Davis medium is given in Table I. The diluted "Tween 80" solution was autoclaved in the same manner as the medium, while the bovine albumin‡ was sterilized by Seitz filtration. All reagents were chemically pure and the pH of the medium was adjusted to 7.2. It was autoclaved at 15 pounds of pressure for fifteen minutes. The medium was incubated for sterility at 37° C. for twenty-four hours before use. All pH determinations were made with the Beckman pH meter. To demonstrate antibiotic activity the scheme of mixing the subtilin solution and the medium, shown in Table II, was adopted. The subtilin solutions were measured by means of

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Part of a paper presented at the meeting of the Society for Experimental Medicine, Dr. Howard D. Lightbody and Associates (who supplied the antibiotic), Laboratory, U. S. Department of Agriculture, Albany, Calif., and Dr. A. C. C. Department of Bacteriology, University of California at Los Angeles.

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†Supplied by Dr. Walter Jamieson, Director of the Biological Division of the Lilly Research Laboratories, Indianapolis, Ind.

‡From The Armour Laboratories, Chicago, Ill.

TABLE IV. RESULTS OF MICROSCOPIC EXAMINATION OF GUINEA PIG TISSUES TO DIFFERENTIATE THE BACTERIOSTATIC AND BACTERICIDAL PROPERTIES OF SUBTILIN

ANIMAL	CONCENTRATION OF SUBTILIN IN INOCULUM	FINDINGS
1 (control)	0	Liver: massive tubercle infiltration Lung: tubercles present throughout
2 (control)*	0	Lymph nodes and spleen: tubercles present Liver: greater involvement than in other tissues
3	1:10,000	No evidence of tuberculosis
4	1:10,000	No evidence of tuberculosis
5	1:20,000	No evidence of tuberculosis
6	1:20,000	No evidence of tuberculosis
7	1:40,000	Lung: numerous early lesions found
8	1:40,000	No evidence of tuberculosis
9	1:80,000	Spleen: tuberculous invasion noted
10	1:80,000	No evidence of tuberculosis
11	1:100,000	Lung: early lesions and areas of consolidation
12	1:100,000	Lung: tubercles present Liver: overwhelming invasion noted Spleen: tubercles present
13	1:200,000	No evidence of tuberculosis
14	1:200,000	Spleen: tubercles present Testis: castration observed

\*Died on the nineteenth day of infection.

1:20,000 dilution or lower of subtilin was definitely bactericidal, while dilutions between 1:40,000 and 1:200,000 of the antibiotic appeared to be bacteriostatic rather than bactericidal. This was substantiated by the finding that five of eight animals inoculated with cultures containing these dilutions revealed various stages of tuberculous infections when animals were sacrificed four weeks after initial infection. In the third place, infections among the experimental animals (Animals 3 to 14) on the whole were less severe than in the controls. This would further indicate that the number of organisms originally present was small since microscopic examinations did not reveal their presence.

A comparison was made between the tuberculin skin test and the macroscopic and the histologic findings in guinea pigs infected with tuberculosis. It was found that histologic studies gave the highest number of positives. Since only one dose of tuberculin was used it is conceivable that animals with early, mild infections may not develop allergy to a degree detectable by 0.01 mg. of P. P. D. In general there is close agreement among the three tests.

#### COMMENT

The use of modified Dubos and Davis medium has permitted the rapid evaluation of an antibiotic, subtilin, against a virulent strain of *Mycobacterium tuberculosis*, the result of which has, heretofore, been inconclusive. This was effected by incorporating a synthetic wetting agent, "Tween 80," and by the addition of a purified albumin fraction to a simple synthetic medium. The former made possible submerged growth while the latter increased the growth rate of the acid-fast bacilli, all of which favors subtilin standardization.

Although subtilin regularly inhibited the growth of our strain of *Mycobacterium* in concentration of 1:400,000, subsequent animal inoculation revealed

that its bactericidal property in vitro was low. It required a concentration of 1:20,000 to sterilize the organisms employed in these tests. It may be inferred, therefore, that the chief mode of action of this antibiotic in vivo is bacteriostatic since the concentration of this amount in the blood stream or tissues can hardly be achieved. Unpublished data from this laboratory indicate that subtilin is precipitated in the presence of sodium chloride. Concentrations of subtilin (University of California Lots 8 and 10) greater than 0.1 mg. per cent appear to be precipitated in the blood. Nevertheless, the marked bacteriostatic effect shown here in conjunction with the low toxicity of subtilin are favorable indications for therapeutic trials. A less toxic salt-soluble fraction of subtilin is now available for such trials.<sup>7</sup>

#### SUMMARY

The modified Dubos and Davis medium is suitable for demonstrating the antibiotic activity of subtilin against a virulent human strain of *Myco. tuberculosis*. Under the conditions of the test a 1:400,000 dilution of subtilin regularly inhibited the growth of these organisms, while dilutions of 1:20,000 or lower were required to achieve bactericidal effect.

#### REFERENCES

1. Salle, A. J., and Jann, G. J.: Subtilin—An Antibiotic Produced by *Bacillus subtilis*: I. Action on Various Organisms, *Proc. Soc. Exper. Biol. & Med.* 60: 60, 1945; II. Toxicity of Subtilin to Living Embryonic Tissue, *Proc. Soc. Exper. Biol. & Med.* 61: 23, 1946; III. Effect on Type III *Pneumococcus* in Mice, *Proc. Soc. Exper. Biol. & Med.* 62: 40, 1946.
2. Lightbody, H. D., and others: Unpublished results.
3. Wong, S. C., and others: Unpublished results.
4. Dubos, R. J., and Davis, B. D.: Factors Affecting the Growth of Tubercle Bacilli in Liquid Media, *J. Exper. Med.* 83: 409, 1946.
5. Randolph, T. G., and Mikell, R. F.: Carbol Fuchsin in Propylene Glycol for Rapid Staining of the Tubercle Bacillus, *Amer. Rev. Tuberc.* 49: 109, 1944.
6. Wong, S. C.: The Relation Between the Growth of *Mycobacterium Tuberculosis* and the Yield of Tuberculin on Synthetic Media, *J. Bact.* 33: 451, 1937.
7. Chin, Y. C.: Biologic Properties of Subtilin in Physiologic Saline Solution, *Federation Proc.* 6: 317, 1947.

# FURTHER OBSERVATIONS ON THE CULTIVATION OF TUBERCLE BACILLI FROM PATHOLOGIC MATERIAL IN DUBOS MEDIA

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## INTRODUCTION

THE bacteriologic diagnosis of tuberculosis still is dependent upon the demonstration of the tubercle bacillus by direct microscopic examination, by animal inoculation, by culture, or by various combinations of these methods. Although the guinea pig remains the method of choice, especially with small specimens or specimens of material which are not easily obtainable, the decreasing use of animal injection has placed more and more emphasis on cultures for the diagnosis of those cases which are negative by direct microscopic examination. In the past years several generally satisfactory methods for the cultivation of *Mycobacteria* have been developed, such as Dorset's,<sup>1</sup> Petragnani's,<sup>2</sup> Löwenstein's,<sup>3</sup> and Schwabacker's<sup>4</sup> variations of coagulated egg media. Most of these, if properly prepared and used with reasonable care, yield a high percentage of positive results. Fundamentally, however, there are two objections which might be made to such media: (1) difficulty in preparation and (2) the time required to obtain growth of tubercle bacilli from pathologic material, often a matter of six or eight weeks.

Dubos<sup>5</sup> recently described a medium which not only supported the growth of minimal inocula of stock strains of tubercle bacilli, but also permitted the rapid growth of cultures planted with such inocula. Foley<sup>6</sup> in a recent study found that such media could be used for the isolation of tubercle bacilli from pathologic material. The purpose of the present study is to report further experience with these media in the bacteriologic diagnosis of tuberculosis.

## METHODS

In the previous study<sup>6</sup> several variations of the basic medium as described by Dubos<sup>5</sup> were employed. Since there appeared to be little difference in the results obtained in these various media, further investigation has been limited to the use of one formula, that most recently described by Dubos<sup>7</sup> (Table I).

The mineral base can be prepared as a stock solution and then stored at room temperature with the addition of a few drops of chloroform, the various solutions (and agar if desired) being added just prior to use. Serum albumin must be inactivated before use in media containing "Tween" since it may contain sufficient lipase to split "Tween" into free fatty acids which inhibit the growth of the tubercle bacillus. Dubos<sup>8</sup> recently suggested the addition of sodium fluoride to the medium to inactivate the serum albumin lipase. Preliminary experiments in this laboratory with the H37RV strain indicate that as little as 0.005 per cent sodium fluoride is sufficient to prevent lipase activity and yet not interfere with the growth of the tubercle bacillus.

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TABLE I. CULTIVATION OF TUBERCLE BACILLI FROM PATHOLOGIC MATERIAL:  
PREPARATION OF MEDIA

Potassium phosphate (Dihydrogen)	1.00 Gm.
Sodium phosphate (Disodium)	6.25 Gm.
Sodium citrate	1.50 Gm.
Magnesium sulfate	0.60 Gm.
"Tween," 80*	5.00 ml. of 10% aqueous solution
Casein hydrolysate	20.00 ml. of 10% neutral solution
Distilled water ad.	1,000.00 ml.
Adjust to pH 7.0-7.2 with normal sodium hydroxide	
Dispense in 5 ml. volumes in properly prepared tubes	
Autoclave at 15 pounds for fifteen minutes	
To each 5 ml. tube of sterile media, add aseptically:	
0.400 ml. of 5 per cent serum albumin in 2 per cent saline†	
0.005 ml. of 10 per cent vegex,‡ autoclaved aqueous solution	
0.050 ml. of 10 per cent glucose, filtered aqueous solution	

\*A polyoxyethylene derivative of sorbitan monooleate, product of Atlas Powder Company, Wilmington, Del. "Tween" must be kept in refrigerator and made up fresh each time medium is prepared. Stored in the mineral base it rapidly splits into products inhibitory to the growth of the tubercle bacillus.

†Bovine fraction V, Armour Laboratories, Chicago, Ill. Desiccated albumin must be kept frozen. Solution prepared fresh in 2 per cent saline for each lot of media, filtered through a Seitz or porcelain filter, and inactivated at 56° C. for thirty minutes.

‡Product of Vegex Corporation, New York, N. Y.

Mineral base may be stored at room temperature with a few drops of chloroform.

"Tween," albumin, glucose, and vegex must be added just prior to use.

All minerals C.P., not recrystallized.

The fluid medium was dispensed in 5 ml. amounts in 7 by 1 Pyrex glass test tubes which previously had been chemically cleaned, rinsed with distilled water, stoppered with cotton, and autoclaved at 15 pounds of pressure for fifteen minutes instead of the usual sterilization by dry heat. The preparation of glassware is most important, since residual soaps, oils rendered from cotton plugs by dry heat, fats, etc., may inhibit the growth of the tubercle bacillus.

Duboss also has described a solid medium prepared by the addition of 1.5 per cent agar-agar and 0.001 per cent ferrie ammonium citrate to the mineral solution with "Tween" (Table I). The mineral base, "Tween," and agar-agar are autoclaved; vegex, glucose, and serum albumin are added aseptically. The agar is dispensed in Petri dishes with moist filter paper attached inside the cover. After inoculation the dish is sealed with adhesive tape to prevent the loss of moisture by excessive evaporation.

For cultural examination, 5 to 20 ml. volumes of the specimens were digested for periods varying from one-half to two hours at room temperature with an equivalent volume of 3 per cent hydrochloric acid, then neutralized to approximately pH 7.0 with 3 per cent sodium hydroxide. The digest was centrifuged and the sediment concentrated in one-tenth to one-twentieth the original volume in sterile physiologic saline. The shorter periods of digestion are desirable since prolonged treatment with acid or alkali undoubtedly kills some of the tubercle bacilli in the sample. Catheterized or bladder urines and gastric aspirations usually can be freed of commensural bacteria by digestion for one-half hour or less, while material such as sputum usually requires longer periods. Media were inoculated with 0.1 to 0.5 ml. of digest and incubated at 37° C. for twenty-one days. Acid-fast smears were examined as soon as visible growth appeared; in any case, on the seventh, fourteenth, and twenty-first days of incubation. The final smear on the twenty-first day was made on the centrifuged sediment of the broth cultures.

Since the previous report,<sup>6</sup> 197 specimens have been examined for tubercle bacilli by these methods as well as by guinea pig inoculation. Of these, twenty-eight were positive either by culture or guinea pig, or both. The results obtained with these positive specimens are summarized in Table II.

TABLE II. CULTIVATION OF TUBERCLE BACILLI FROM PATHOLOGIC MATERIAL: COMPARISON OF GUINEA PIG INOCULATION WITH CULTIVATION IN DUROS' MEDIA

MATERIAL	NUMBER OF POSITIVE SPECIMENS	NUMBER POSITIVE BY		INCUBATION IN DAYS	
		GUINEA PIG	CULTURE	RANGE	MEAN
Biopsy material					
Eye	1	1	1	7	
Lymph nodes	2	2	2	7	9
Miscellaneous	2	2	2	10-14	
Gastric aspiration	2	1	2*	14-21	12
Joint fluid	3	3	3	5-21	15
Urine					
Bladder	5	5	5	7-15	12
Catheterized	2	2	2	7-9	8
Sputum	6	6	4†	7	7
Spinal fluid	5	5	3	10	10
Total	28	27	24	5-21	10.4

\*Patient with positive culture and negative guinea pig not available for further study.

†One of two guinea pigs positive on each of two specimens which were negative by culture.

## RESULTS

As is shown in Table II, twenty-three (82 per cent) of the twenty-eight specimens which contained tubercle bacilli were positive by culture as well as by guinea pig. One additional specimen, a gastric aspiration which was positive by culture, did not produce tuberculosis in guinea pigs. The incubation periods of these positive specimens ranged from five to twenty-one days, with a mean of 10.6 days, remarkably little time for the bacteriologic diagnosis of tuberculosis. The remaining specimens, two sputa and two spinal fluids, were positive by guinea pig only, tubercle bacilli not being found in culture.

The results obtained with this small series of specimens are combined with those previously reported<sup>6</sup> in Table III. Of the fifty-seven specimens which contained tubercle bacilli, fifty (87.7 per cent) were positive by culture, a figure which is not significantly different from the fifty-four (94.6 per cent) specimens which were positive in guinea pigs. The incubation periods for the positive cultures in this larger series ranged from five to twenty-seven days, with a mean of 10.6 days. Forty-four (88 per cent) of the positive results

TABLE III. CULTIVATION OF TUBERCLE BACILLI FROM PATHOLOGIC MATERIAL: RESULTS OBTAINED WITH POSITIVE SPECIMENS

MATERIAL	NUMBER OF SPECIMENS	NUMBER POSITIVE BY		PER CENT POSITIVE BY CULTURE	INCUBATION IN DAYS	
		GUINEA PIG	CULTURE		RANGE	MEAN
Biopsy, surgical, and post-mortem material	7	7	7	100.0	6-14	10.5
Gastric aspiration	8	7*	8	100.0	7-27	12.0
Joint fluid	5	5	5	100.0	5-21	14.3
Urine	14	14	13†	92.8	7-20	11.4
Sputum	16	14‡	13†	81.2	5-21	7.6
Spinal fluid	7	7	4	57.1	6-10	8.0
Total	57	54	50	87.7	5-27	10.6

\*Patient with negative guinea pig and positive culture not available for further study.

†One guinea pig positive on case with negative culture.

‡Two sputa negative in guinea pigs and positive by culture were from known cases of tuberculosis.

were obtained with incubation periods of fourteen days or less, a sharp contrast to the eight to twelve weeks usually required for diagnosis by guinea pig inoculation.

As yet, too few positive specimens have been studied on the solid medium to permit analysis. The few strains isolated as well as the stock cultures of tubercle bacilli grew as small, smooth, soft, colorless, semitransparent colonies after seven to ten days of incubation. Colony morphology was not unlike that of the streptococcus, markedly different from the hard, granular, opaque growth usually observed in coagulated egg media. Four spinal fluids which failed to grow in broth yielded positive results within ten days when cultivated on the agar medium.

#### DISCUSSION

The specimens (Table IV) with which divergent cultural and guinea pig results were obtained are of particular interest. A positive cultural result with those specimens which were negative by guinea pig suggests either of two explanations: such results merely represent differences in the number of tubercle bacilli in the inocula used for culture and guinea pig or these strains were avirulent. In the case of the two sputa, the former seems the most likely since both specimens were obtained from patients known to have tuberculosis. In the case of the gastric aspiration, repeat specimens for further study were unobtainable and it could not be established whether the patient did or did not have tuberculosis. The microorganism isolated had the cultural and growth characteristics of tubercle bacilli but was avirulent for guinea pigs.

TABLE IV. CULTIVATION OF TUBERCLE BACILLI FROM PATHOLOGIC MATERIAL: DISCREPANT RESULTS BETWEEN GUINEA PIG INOCULATION AND CULTIVATION IN DUBOS' MEDIA

MATERIAL	TOTAL NUMBER OF SPECIMENS	NUMBER OF SPECIMENS		
		POSITIVE G.P. POSITIVE CULTURE	POSITIVE G.P. NEGATIVE CULTURE	NEGATIVE G.P. POSITIVE CULTURE
Gastric aspiration	8	7	0	1
Urine	14	13	1	0
Sputum	16	11	3	2
Spinal fluid	7	4	3	0

A positive guinea pig and a negative culture on the same specimen also is amenable to either of two explanations. If the material contained but a few tubercle bacilli, it is conceivable that acid digestion might reduce the number of viable cells below the minimum necessary for growth in artificial media. The alternate explanation is the probability that certain strains fail to grow in these media. Dubos<sup>5</sup> feels that the medium does not yet provide the optimal factors essential for the growth of all strains of tubercle bacilli. That there are differences between various strains is well illustrated by the four spinal fluids which were negative in fluid media but yielded prompt positive results when cultured on Dubos' agar.

Saprophytic Mycobacteria as yet cannot be sufficiently differentiated to permit the exclusive use of these media for the specific diagnosis of tuber-

culosis on certain kinds of material; namely, urine and gastric aspiration. However, in those instances where further bacteriologic identification is indicated, the use of an isolated culture for guinea pig injection affords a considerable saving of time.

#### SUMMARY

The fluid and solid media recently described by Dubos have been successfully applied to the rapid cultivation of tubercle bacilli from pathologic material.

Of fifty-seven positive specimens examined in fluid media, fifty (87.7 per cent) were positive by culture, while fifty-four (94.6 per cent) were positive by guinea pig inoculation. Incubation periods for the positive cultures ranged from five to twenty-seven days, with a mean of 10.6 days. Forty-four (88 per cent) of the positive results were obtained within fourteen days of incubation.

Too few specimens have been examined on the solid medium to permit analysis. Four strains which failed to grow in broth grew readily on the solid medium. Colony morphology on this medium is strikingly different from that observed on coagulated egg media. Growth occurs as soft, discrete, semitransparent colonies not unlike those of the streptococci.

#### REFERENCES

1. Dorset, M.: The Use of Eggs as a Medium for the Cultivation of *Bacillus tuberculosis*, *Am. Med.* 3: 555-556, 1902.
2. Cerruti, C. F.: The Culture of *B. tuberculosis* on Petragnani's Medium, *J. Trop. Med.* 35: 157-158, 1932.
3. Löwenstein, E.: La Methode d'Hémoculture du Virus Tuberculeux et ses Résultats, *Ann. Inst. Pasteur* 50: 161-166, 1933.
4. Schwabacker, H.: A Comparison of Different Media for the Growth of Tubercle Bacillus, *Tubercle* 18: 199-205, 1937.
5. Dubos, R. J.: Rapid and Submerged Growth of Mycobacteria in Liquid Media, *Proc. Soc. Exper. Biol. & Med.* 58: 361-362, 1945.
6. Foley, G. E.: Submerged Growth of Tubercle Bacilli From Pathologic Material in Dubos' Medium, *Proc. Soc. Exper. Biol. & Med.* 62: 298-302, 1946.
7. Dubos, R. J., and Davis, B. D.: Factors Affecting the Growth of Tubercle Bacilli in Liquid Media, *J. Exper. Med.* 83: 409-423, 1946.
8. Dubos, R. J.: Personal communications to this laboratory.

# DISCREPANCIES IN THE AGGLUTINATION TEST FOR BRUCELLOSIS AS PERFORMED WITH VARIOUS ANTIGENS AND AS REPORTED FROM DIFFERENT LABORATORIES

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**I**NCONSISTENCIES in the results of brucella agglutination tests when performed on the same serum with different antigens have been the cause of concern in our laboratory. Although close attention was given to uniformity and accuracy of the technical details of the test, nevertheless, significant differences in titer have been observed with disconcerting frequency when several commercial antigens were used in routine testing. Furthermore, results obtained with different lots of the same commercial antigen were not infrequently at variance with each other.

Similar experiences have been noted by others. Serum from a patient with culturally proved chronic brucellosis reported by Spink, Hall, and Aagaard<sup>1</sup> was sent to two different laboratories for agglutination tests. One laboratory reported that no brucella agglutinins were present; the other reported an agglutinin titer of 1:400. Angle, Algie, and Morgan<sup>2</sup> observed similar discrepancies and cite an example in which a serum was reported by a state laboratory as giving a negative test for brucella agglutinins, but on which positive tests were obtained by them in dilutions of 1:640 with three commercial antigens.

The present study was undertaken to explore the extent of the variability of brucella agglutinin titers as determined with different antigens and also as reported by different laboratories. It is an attempt to determine the deviations one might expect to encounter in actual practice under existing laboratory conditions.

## PROCEDURE

Fifteen milliliters of serum were collected from each of eighteen individuals known to have moderate brucella agglutinin titers. In seven of the patients, blood cultures had been positive for *Brucella abortus* from sixteen months to seven years before, but the infection was considered to be quiescent at the time of the present tests. In one individual, brucella agglutinins had developed in response to vaccination for cholera.<sup>3</sup> The other ten were presumed either to be suffering from chronic brucellosis or to have recovered from the disease. In addition, sera were collected from three rabbits, each of which had been infected with one of the three species of *Brucella*—*melitensis*, *abortus*, and *suis*. Because of the very high agglutinin titers of these rabbit sera, dilutions were made with normal rabbit serum to bring the titers into a more moderate range.

The opsonocytophagic test was done on the blood of each patient at the time the serum was collected. In the blood of fifteen of the patients, there was marked phagocytosis by 80 per cent or more of the leucocytes, while the other three showed moderate phagocytosis. This tends to confirm further the specific identity of the antibody response in these patients.

Each serum was collected and prepared aseptically and was divided into six lots in sterile, stoppered tubes. One tube of each serum was sent to each of five laboratories other

From the Department of Medicine, the University of Chicago.  
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TABLE I. THE RESULTS OF BRUCELLA AGGLUTINATION TESTS PERFORMED ON

SERUM	TESTS IN OUR LABORATORY									
	TEST TUBE ANTIGENS			RAPID SLIDE ANTIGENS						
	1	2	3	4	5	6	7	8	9	10
1. N. C.	1/160	1/320	1/160	1/160	1/200*	1/100	1/400*	1/200	1/100	1/200
2. M. A.	1/160	1/320	1/160	1/160	1/200*	1/100†	1/200	1/200	1/100	1/200†
3. D. S.	1/160	1/160	1/80	1/160	1/200*	1/100	1/400†	1/100	1/100	1/100
4. S. L.	1/160	1/320	1/320	1/160	1/200*	1/200†	1/400*	1/200	1/100	1/200†
5. S. B.	1/80	1/160	1/80	1/80	1/100	1/50	1/100	1/100	1/50	1/50
6. M. A.	1/320†	1/320	1/160	1/160†	1/100	1/100†	1/200	1/100	1/50	1/100
7. G. F.	1/80	1/160	1/80	1/160	1/100	1/100	1/400*	1/200†	1/50	1/50
8. J. B.	1/1280			1/320*	1/200*	1/500*	1/400*	1/400*	1/400*	1/400*
9. C. C.	1/320†	1/640	1/320	1/320*	1/200*	1/200†	1/400*	1/200	1/100	1/100
10. A. B.	1/40	1/80	1/40	1/80	1/50	1/25	1/100	1/50†	1/50	1/50
11. W. F.	1/320†	1/320		1/160	1/200*	1/100	1/400*	1/200	1/100	1/200
12. M. F.	1/80	1/160	1/80	1/80	1/50	1/25	1/100	1/50	1/50	1/50
13. P. M.	1/160	1/640	1/160	1/160	1/200*	1/100	1/400*	1/100	1/50	1/100
14. J. S.	1/640†	1/1280	1/640	1/160	1/200*	1/100	1/400*	1/200	1/100	1/200†
15. W. T.	1/320	1/1280	1/640	1/320*	1/100	1/100	1/400*	1/200	1/50	1/100
16. P. V.	1/320	1/640	1/320	1/160	1/200*	1/100	1/400*	1/200†	1/100	1/200
17. J. W.	1/20			1/40	1/25†	1/25	1/100	1/25	Neg.	1/25
18. J. G.	1/160		1/160	1/160†	1/100	1/50†	1/200	1/100	1/100	1/200†
19. Rabbit—Br. abortus	1/640				1/200*		1/400*	1/400*	1/100	1/500
20. Rabbit—Br. melitensis	1/640				1/200*		1/400*	1/50	1/50	1/100
21. Rabbit—Br. suis	1/320				1/200*		1/400*	1/100	1/100	1/200

\*The maximum titer obtainable with this antigen.

†Partial agglutination but agglutination usually complete in the next lower dilution.

than our own with a request for routine brucella agglutination tests. No information was given to the laboratory concerning the clinical histories, nor were the reasons for requesting these tests divulged. Two public health laboratories, two private clinical laboratories, and one veterinary laboratory were selected as representative of those commonly used in practice.

The sixth tube of each serum was tested in our own laboratory against the following ten antigens: seven commercial rapid slide antigens, one commercial test tube antigen, one test tube antigen supplied by the Bureau of Animal Industry of the U. S. Department of Agriculture, and one test tube antigen prepared in our laboratory from *Br. abortus* strain 295 in accordance with the directions of Huddleson.<sup>5</sup> The eight commercial antigens were purchased on the open market and represented all those which were available to us after a thorough search of sources. Several of the specimens were not tested with all the antigens because of insufficient sera. All tests performed in our laboratory were in accordance with standard techniques.<sup>7</sup>

As a matter of interest, sera were similarly collected from three patients who had recently received intravenous combined typhoid vaccine for fever therapy. These sera were tested for typhoid agglutinins in our laboratory and were also submitted to five other laboratories. The same laboratories were used as for the brucella agglutination tests, except that a hospital laboratory was substituted for the veterinary laboratory.

## RESULTS

The results of the brucella agglutination tests performed in our laboratory as well as those reported from the five other laboratories are presented in Table I. The table also gives the maximum and minimum values reported for each serum by the five laboratories as well as the maximum and minimum values of all fifteen tests.

THE SAME SERUM WITH DIFFERENT ANTIGENS AND IN DIFFERENT LABORATORIES

TESTS IN OTHER LABORATORIES					RANGE OF ALL 15 TESTS		RANGE OF REPORTS FROM OTHER LABORATORIES	
PUBLIC HEALTH LABORA- TORY	PUBLIC HEALTH LABORA- TORY	PRIVATE CLINICAL LABORA- TORY	PRIVATE CLINICAL LABORA- TORY	VETERI- NARY LABORA- TORY			MAXI- MUM	MINI- MUM
A	B	C	D	E	MAXI- MUM	MINI- MUM	MAXI- MUM	MINI- MUM
1/160	1/160	1/200	1/80	1/320	1/100	1/80	1/320	1/80
1/640	1/160	Neg.	1/10	Neg.	1/640	Neg.	1/640	Neg.
1/320	1/80	Neg.	Neg.	Neg.	1/100†	Neg.	1/320	Neg.
1/160	Neg.	1/200	1/80	1/80	1/100	Neg.	1/200	Neg.
1/160	Neg.	Neg.	1/40†	Neg.	1/160	Neg.	1/160	Neg.
1/320	1/160	Neg.	1/80	Neg.	1/320	Neg.	1/320	Neg.
1/160	Neg.	1/200	1/40	1/80	1/100	Neg.	1/200	Neg.
1/1280	1/1280	1/500*		1/320	1/1280	1/320	1/1280	1/320
1/320	1/80	1/200	Neg.	1/160	1/640	Neg.	1/320	Neg.
Neg.	Neg.	Neg.	1/200	Neg.	1/200	Neg.	1/200	Neg.
1/640	1/160	1/100	Neg.	1/40	1/640	Neg.	1/640	Neg.
Neg.	Neg.	1/100	Neg.	Neg.	1/100	Neg.	1/100	Neg.
1/320	Neg.	1/100	Neg.	1/40	1/640	Neg.	1/320	Neg.
1/640	Neg.	1/200	Neg.	1/80	1/1280	1/20	1/640	Neg.
1/160	1/80	1/200	1/20	1/80	1/1280	Neg.	1/200	1/20
1/640	1/160	1/200	Neg.	1/80	1/640	Neg.	1/640	Neg.
Neg.	Neg.	Neg.	Neg.	Neg.	1/100	Neg.	Neg.	Neg.
1/50	Neg.	1/100	1/20		1/200	Neg.	1/100	Neg.
1/640	1/320	1/500*	Neg.		1/640	Neg.	1/640	Neg.
1/640	1/320	1/100	Neg.	Neg.	1/640	Neg.	1/640	Neg.
	1/320	1/500*	Neg.	1/80	1/500	Neg.	1/500	Neg.

In the titers reported from the five laboratories other than our own, it will be noted that of the twenty-one sera submitted, eighteen were called negative by one or more laboratories. Of these eighteen "negative sera," all except one were reported by other laboratories as positive in titers ranging from 1:100 to 1:640.

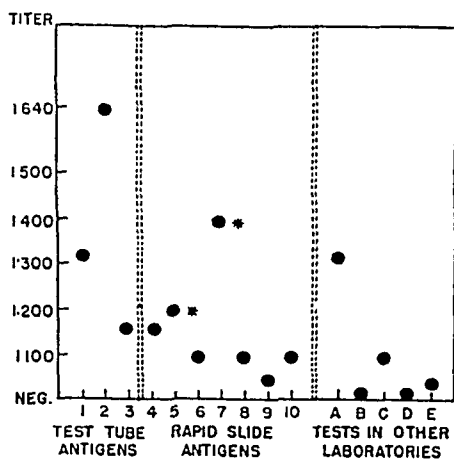
The variations of the agglutinin titers obtained in our own laboratory with the ten different antigens confirmed, in a general way, and, in a few cases, extended the magnitude of the deviations reported by the other laboratories. In our laboratory, however, only one serum was found to be negative in any test.

The variations in the brucella agglutination tests of six selected cases are presented graphically in Fig. 1.

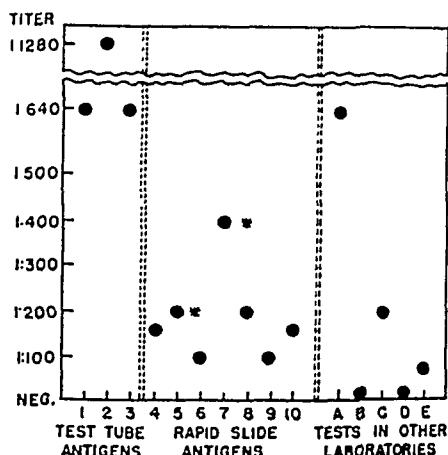
The typhoid and paratyphoid agglutination tests that were reported on the sera from three patients treated with intravenous typhoid combined vaccine are presented in Table II. Inconsistencies in the titers reported by different laboratories are evident in these data also.

#### DISCUSSION

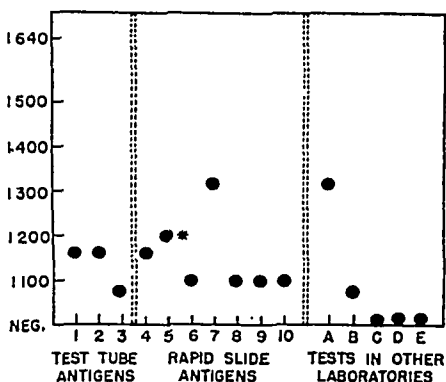
The brucella agglutination test is more widely used than any other procedure in the diagnosis of brucellosis. It is used extensively in the diagnosis of the human disease as well as being the generally accepted criterion for the presence of infection in cattle. At the first Inter-American Brucellosis Congress held in Mexico City in 1946, various speakers indicated that "the agglu-



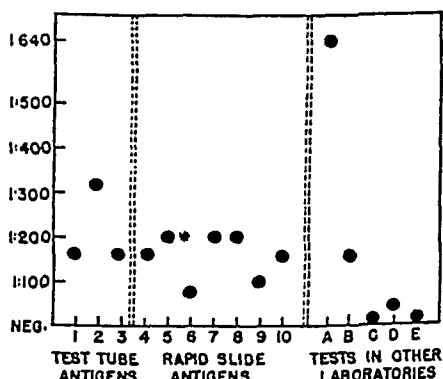
CASE P.M. ACUTE BRUCELLOSIS WITH BLOOD CULTURES POSITIVE FOR BR. ABORTUS 26 MONTHS BEFORE. PROMPT AND COMPLETE CLINICAL RECOVERY WITHOUT RELAPSE.



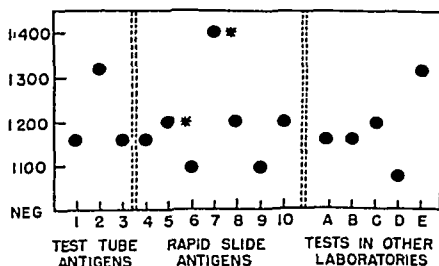
CASE J.S. ACUTE BRUCELLOSIS WITH BLOOD CULTURES POSITIVE FOR BR. ABORTUS 26 MONTHS BEFORE. PROMPT AND COMPLETE CLINICAL RECOVERY WITHOUT RELAPSE.



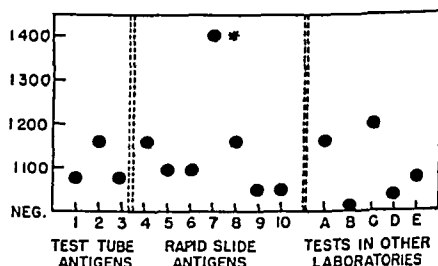
CASE D.S. "PROBABLE" CHRONIC BRUCELLOSIS



CASE M.A. "PROBABLE" CHRONIC BRUCELLOSIS



CASE N.C. CHRONIC BRUCELLOSIS ACUTE ILLNESS WITH BLOOD CULTURES POSITIVE FOR BR. ABORTUS 2 YEARS BEFORE.



CASE G.F. CHRONIC BRUCELLOSIS (ARRESTED?), BLOOD CULTURES POSITIVE FOR BR. ABORTUS 16 MONTHS BEFORE.

\* THE MAXIMUM TITER OBTAINABLE WITH THIS ANTIGEN

Fig. 1.—Variations in the results of brucella agglutination tests on the same serum when performed with various antigens and when reported from different laboratories.



TABLE II. AGGLUTINATION TESTS FOR TYPHOID AND PARATYPHOID: RESULTS OF TESTS ON THE SAME SERUM REPORTED FROM DIFFERENT LABORATORIES

PATIENT	ANTIGEN	OUR LABORATORY		OTHER LABORATORIES				
		TEST TUBE ANTIGEN	PLATE ANTIGEN	PUBLIC HEALTH LABORATORY A	PUBLIC HEALTH LABORATORY B	PRIVATE CLINICAL LABORATORY C	PRIVATE CLINICAL LABORATORY D	HOSPITAL LABORATORY F
1 (A. F.)	Typhoid		1/320*	Neg.			1/500*	
	Typhoid O				Neg.	1/400		1/640
	Typhoid H				1/1280	1/1000		1/1280
	Paratyphoid A				Neg.	1/500		1/2560
	Paratyphoid B				1/640	1/500		1/2560
2 (V. F.)	Typhoid	1/100	1/320*	1/160		1/200	1/80	1/640
	Typhoid O	1/50			Neg.	1/400	1/160	1/640
	Typhoid H	1/250			1/80	1/200	1/160	1/640
	Paratyphoid A				1/160	1/400	1/160	1/640
	Paratyphoid B				1/320		1/160	1/640
3 (H. W.)	Typhoid	1/2500	1/320*	1/640		1/300	1/640	1/2560
	Typhoid O	1/1000			Neg.	1/2000	1/320	1/2560
	Typhoid H	1/1000			1/320		1/320	1/2560
	Paratyphoid A				1/1280	Neg.	1/640	1/2560
	Paratyphoid B				1/1280	Neg.		1/2560

\*The maximum titer obtainable with this antigen.

ination test continues to be the most useful procedure in the diagnosis of active brucellosis.<sup>14</sup> It is therefore of considerable importance to evaluate the reliability and accuracy of the test as it is applied in actual practice.

The significance of the agglutination test in relation to human brucellosis has long been subject to discussion, and its interpretation in terms of clinical infection may be a difficult task indeed. The question is frequently asked, what is a clinically significant titer? An unqualified answer cannot be given and a number of limitations must be considered.

Several sources of difficulty in the interpretation of the brucella agglutination test arise from biologic factors. It is well known that individuals vary markedly in their ability to produce brucella agglutinins in response to clinical infection. Also, different strains of the organism vary in their agglutinogenic power. Many authors<sup>1, 2, 5</sup> have reported cases of brucellosis verified by blood cultures in which no serum agglutinins appeared. Conversely, apparently healthy individuals with significant titers in their blood are found in brucella-agglutinin surveys. Thus, blood from a patient who has brucellosis may contain no agglutinins, while blood from a symptomless individual may contain them.

It has long been known that infection with *Pasturella tularensis* produces a cross-agglutination response for brucella. Recently, we have shown that significant titers of brucella agglutinins, as well as opsonins, develop in most individuals receiving the standard two-dose cholera vaccination.<sup>3</sup> This response is due to an H antigen of *Vibrio comma*.

In addition to the irregularities of agglutinin response arising from the uncontrollable biologic variables, the present studies demonstrate that the measurement of brucella agglutinins in any given serum is subject to great variation with the usual laboratory methods. The magnitude of this variation is such that of twenty-one sera submitted to five qualified laboratories for routine test-

ing, reports on each of eighteen varied from completely negative to positive in dilutions of 1:100 to 1:640. Two-thirds of the so-called "negative tests" were reported elsewhere as positive in dilutions of 1:320 or 1:640—titers which are considered by many physicians as substantiating a clinical diagnosis of brucellosis. Similarly, a negative test is often regarded as excluding that diagnosis. Depending on which laboratory report is accepted as the actual titer, at least two-thirds of the cases of this series can be thus either "ruled out" or "substantiated." That such blind reliance on agglutination tests is deplorable is well demonstrated by these data. Our views on the clinical interpretation of the agglutination test have been discussed in previous publications.<sup>6</sup>

We do not intend to imply that the brucella agglutination test is a useless procedure. If one is not indifferent to the sources of error and if one does not attempt to interpret the test too strictly, it may be a useful adjunct to diagnosis. We believe that the variations observed in this study are mainly due to a lack of standardization of the antigens. Angle, Algic, and Morgan<sup>2</sup> noted similar diversity in agglutination titers when they used antigens which they prepared from twenty stock strains of brucella. Variations of technique of the test may also be a contributing factor. The admonition of the Committee on Bang's Disease of the United States Live Stock Sanitary Association<sup>7</sup> bears repeating: "The reading of tests should be carried on only by individuals who have had wide experience in this work. Extreme care should be exercised in the observation of tests to minimize the discrepancies resulting from improper or careless observation." It would appear that much of the difficulty could be avoided if standardization of all antigens were supervised by some central agency, or if standard sera were available for checking antigens. The test as used in our laboratory has given consistent results when antigens prepared from the same strain have been employed.

Limited observations on the results of typhoid and paratyphoid agglutination tests made on sera from patients treated with typhoid vaccine suggest that the variations observed in the agglutination test for brucella also occur in other agglutination tests. It is noteworthy that the agglutinin response in these patients was the result of vaccine therapy rather than of a natural infection, as was the case in the brucella sera. It should also be mentioned that inconsistencies in the testing of typhoid and paratyphoid agglutinins are of considerably less clinical importance than in the case of brucella agglutinins. In comparison with brucellosis, the agglutination test plays a relatively minor role in the diagnosis of the enteric infections.

#### SUMMARY

1. Wide variations in brucella agglutinin titers were observed in each of twenty-one sera tested with ten different antigens in our laboratory. Tests on the same sera made in five other laboratories showed equally great discrepancies.

2. Of the twenty-one sera tested, eighteen were called negative by one or more laboratories. Of these eighteen "negative sera," sixteen, or 89 per cent, were elsewhere called positive in titers of 1:200 or higher; twelve, or

67 per cent, were called positive in dilutions of 1:320 or 1:640—titers which are frequently considered as substantiating a clinical diagnosis of brucellosis.

3. Depending on which laboratory report is accepted as the actual titer, two-thirds of the eighteen cases can be either "ruled out" or "substantiated" by commonly accepted but deplorable practices of interpretation of the test.

4. Limited observations on typhoid and paratyphoid agglutination tests suggest that the variations observed in the tests for brucella also occur in other tests.

#### REFERENCES

1. Spink, W. W., Hall, W. H., and Angaard, G. U.: Chronic Brucellosis, *Staff Meet. Bull., Hosp. of Univ. Minn.* 17: 194, 1946.
2. Angle, F. E., Algie, W. H., and Morgan, D.: Brucellosis: Studies Emphasizing Strain Variation in Serologic Testing, *J. LAB. & CLIN. MED.* 27: 1259, 1942.
3. Eisele, C. W., McCullough, N. B., Beal, G. A., and Burrows, W.: Development of Brucella Agglutinins in Humans Following Vaccination for Cholera, *Proc. Soc. Exper. Biol. & Med.* 61: 89, 1946; also unpublished data.
4. Foreign Letters: *J. A. M. A.* 133: 798, 1947.
5. Huddleson, I. F.: Brucellosis in Man and Animals, New York, 1943, The Commonwealth Fund, pp. 217-219.
6. Eisele, C. W.: Some Problems in the Diagnosis of Chronic Brucellosis, *M. Clin. North America* 31: 182, 1947.
7. Report of Committee on Bang's Disease, Part I: Report of the 35th Annual Meeting of the U. S. Live Stock Sanitary Association, *J. Am. Vet. M. A.* 80: 323, 1932.

# HUMAN BRUCELLA MELITENSIS INFECTIONS IN MINNESOTA WITH HOGS AS THE PROBABLE SOURCE

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THREE species of the genus *Brucella*, *abortus*, *melitensis*, and *suis*, are recognized as the etiologic agents causing brucellosis in man and animals. *Brucella abortus* and *Brucella suis*, etiologic agents for brucellosis in cattle and hogs, respectively, have been recognized in Minnesota since laboratory diagnosis was begun. The *melitensis* species, usual incitant of brucellosis in sheep and goats, has not previously been established as present in man or livestock in this state.

Kabler and MacLanahan<sup>1</sup> in 1936 reported on forty strains of *Brucella* isolated in Minnesota from human cases of brucellosis. They classified these strains as follows: twenty-five strains of *Br. suis*, thirteen strains of *Br. abortus*, and two strains with conflicting reactions, one of which appeared to be *Br. suis* and the other *Br. melitensis*.

Jordan and Borts<sup>2</sup> in 1946 reported the isolation of twenty-six strains of *Br. melitensis* in Iowa from human cases of brucellosis. One strain was isolated in 1930, and twenty-five strains have been isolated since December, 1943.

The present report, based on laboratory findings and epidemiologic investigations made in 1945-1946 by the Minnesota Department of Health, deals primarily with thirteen strains of *Br. melitensis* isolated from human cases of brucellosis.

## MATERIALS AND METHODS

**Blood Culture.**—Twenty-five milliliters of bacto-tryptose broth, pH 6.8, containing 1 per cent sodium citrate, were placed in a 2-ounce rubber diaphragm screw-capped bottle and sterilized. A lead foil cover was placed over the cap and crimped tightly around the neck of the bottle. The bottle was then mailed to the physician with the instructions to draw, aseptically, 5 ml. of blood from the patient during a febrile attack, to insert the needle through the diaphragm of the cap and expel the blood from the syringe into the broth, replace the lead foil over the cap of the bottle, and return the bottle to the laboratory by mail.

Upon receipt of the culture in the laboratory, approximately 10 per cent of the air was aseptically removed from the bottle and replaced with an equal volume of CO<sub>2</sub>. The broth culture was then incubated at 37° C. After five days of incubation, four tubes of bacto-tryptose agar, pH 6.8, were inoculated, each tube receiving approximately 1 ml. of broth culture. Two tubes were incubated aerobically at 37° C. and two tubes in an atmosphere of 10 per cent CO<sub>2</sub>. The broth culture was returned to the 37° C. incubator for another ten days, after which time four more tryptose agar slants were inoculated and incubated as previously described. Each set of subcultures was observed every other day for a period of two weeks. No blood cultures were discarded as negative before thirty days.

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*Brucella* species were identified according to their CO<sub>2</sub> requirements, staining reactions, fermentation reactions, bacteriostatic action of dye plates, H<sub>2</sub>S formation as described by Huddleson and co-workers,<sup>3</sup> and serologic tests.

*Agglutinations.*—Macroscopic tube agglutination tests for the presence of *Brucella* agglutinins were made with serum obtained from clotted whole blood specimens submitted by the attending physician.

#### LABORATORY AND EPIDEMIOLOGIC DATA

During the years of 1945 and 1946, a total of 193 *Brucella* strains were recovered from human sources. Of these strains, 165 (84.5 per cent) were *Br. abortus*, 15 (7.8 per cent) were *Br. suis*, and 13 (6.7 per cent) were *Br. melitensis*.

The results of the laboratory tests and the epidemiologic findings of the thirteen human cases of brucellosis due to *Br. melitensis* are summarized in Table I.

TABLE I. THIRTEEN CASES OF HUMAN BRUCELLOSIS DUE TO *Br. melitensis*: SUMMARY OF LABORATORY FINDINGS AND EPIDEMIOLOGIC DATA

CASE	INITIALS	AGE	AGGLUTINATION REACTION		POSITIVE BLOOD CULTURES DATE RECEIVED	ANIMAL CONTACT		PROBABLE SOURCE OF INFECTION
			DATE RECEIVED	TITER		HOGS	CATTLE	
1	G. D.	53	2/13/45	1:2560	2/16/45	Yes	No	Packing plant, handling hog salvage
2	C. H.	49	3/26/45	1:2560	3/28/45	Yes	No	Packing plant, handling hog casings
3	W. F.	40	3/31/45	1:320	4/ 3/45	Yes	No	Packing plant, handling hog casings
4	M. P.	19	4/30/45	1:5120	5/23/45	Yes	No	Packing plant, handling hog casings
5	W. K.	50	5/10/45	1:1280	5/13/45	Yes	No	Packing plant, lard making
6	K. D.	29	6/ 8/45	1:5120	6/21/45	Yes	No	Packing plant, hog kill
7	R. B.	20	6/21/45	1:1280	6/24/45	Yes	No	Packing plant, hog kill
8	L. J.	29	7/13/45	1:640	7/20/45	Yes	No	Packing plant, pork trim
9	R. C.	25	9/11/45	1:160	9/20/45	Yes	No	Packing plant, shackling hogs
10	C. H.	34	5/ 2/46	1:5120	5/15/46	Yes	Yes	Farm abortion in hogs (cows nonreactors)
11	I. S.	51	6/19/46	1:2560	6/21/46	Yes	No	Packing plant, hog kill
12	L. H.	30	7/ 6/46	1:5120	7/14/46	Yes	Yes	Farm, handling aborted material from hogs (no history of abortion in cattle)
13	R. D.	35	7/30/46	1:5120	8/ 3/46	Yes	No	Packing plant, shackling hogs

All the cases presented in Table I are men; the youngest was 19 years of age, and the oldest 53 years. All had symptoms consistent with the diagnosis of brucellosis. Eleven of the thirteen patients were packing plant employees who handled either live hogs or pork products. Six of the employees handled the hogs at the time of killing, and four handled the pork within forty-five minutes of the killing. One employee, a pork trimmer, handled the pork the day after the animal was killed.

It is noteworthy that all the thirteen patients were residents of four Minnesota counties that border the northern section of Iowa. Two were farmers,

both of whom owned hogs bred and raised locally which aborted. There was no history of abortion in the cattle on either of these farms. Unpasteurized cow's milk was used by two of the thirteen patients; and one obtained raw milk from a cow that gave a negative reaction when tested for Bang's disease.

The hogs handled at the packing plant all came from Minnesota or from points in Iowa, and North and South Dakota, which are readily accessible by truck and rail to the Minnesota market.

#### COMMENTS

The isolation of *Br. melitensis* from the blood of persons in southern Minnesota demonstrates this organism as being endemic in this area.

*Br. melitensis* was originally regarded as the etiologic agent of brucellosis in sheep and goats, and it was believed that these animals act as reservoirs for this organism; however, there is evidence<sup>4, 5</sup> that all species of *Brucella* may infect cows, hogs, sheep, or goats. That hogs are susceptible to *Br. melitensis* is indicated in the statement by Huddleson<sup>3</sup>: "Melitensis is pathogenic for the hog. While I was in Malta in 1938, I saw evidence of its pathogenicity. A considerable amount of left-over unpasteurized goat's milk was fed to a group of pregnant sows. All of them aborted. *Melitensis* was recovered from the fetuses. For some reason this observation was never reported in the literature."

In the series of cases presented in this report, there is no history of contact with sheep or goats. The epidemiologic findings show that all patients had direct contact with hogs; in two cases there was contact with cattle in which there was no history of Bang's disease.

#### SUMMARY

1. *Br. melitensis* has been isolated from blood cultures of thirteen human cases of brucellosis.

2. All the patients were men having direct contact with hogs; eleven were packing plant employees and two were farmers.

3. Epidemiologic studies of *Br. melitensis* infections incriminate hogs as the source of infection; in at least two of the cases, the hogs were bred and raised in Minnesota.

4. All human cases of brucellosis due to *Br. melitensis* that have been detected to date are residents of four counties in south-central Minnesota.

#### REFERENCES

1. Kabler, P., and MacLanahan, M.: A Differential Study of Forty *Brucella* Strains Isolated in Minnesota, *J. Infect. Dis.* 58: 293-298, 1936.
2. Jordan, C. F., and Borts, I. H.: Occurrence of *Brucella melitensis* in Iowa, *J. A. M. A.* 130: 72-75, 1946.
3. Huddleson, J. F., Hardy, A. V., Debono, J. E., and Giltner, Ward: *Brucellosis in Man and Animals*, ed. 2, New York, 1943, Commonwealth Fund, Division of Publications.
4. Topley, W. W. C., and Wilson, G. S.: *The Principles of Bacteriology and Immunity*, ed. 2, Baltimore, 1936, William Wood & Co.
5. Huddleson, Forrest I.: Quoted by Jordan and Borts.<sup>2</sup>

# THE DIFFRACTION METHOD OF MEASURING RED BLOOD CELLS

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THE history of the diffraction method of measuring red blood cells is a story of a lucky accident. It is only because of a mistake by a technical assistant that the method is in use today and has been able to make its contribution to medical science both by stimulating investigations into the diameters of red cells and as a diagnostic method. In 1918 I planted a drop of centrifuged urine into clear broth. Next morning there was a faint haziness, indicating bacterial growth, but on shaking the bottle, a small number of glittering small particles became detached from the bottom. They whirled through the liquid and exhibited a bluish-greenish radiance. Thinking I had found a new color-producing microbe, I made subcultures on agar. A variety of colonies developed, none of them obviously colored. One evening, having another look at the colonies, I moved the Petri dish up and down in front of an electric light bulb. It was then that I noticed that some colonies exhibited a curious colored gloss, in which bluish green predominated, with a faint suggestion of the other colors of the spectrum. (This phenomenon, once one knows of it, can easily be seen in thin surface cultures of many bacteria if one holds the culture between the eye and a bright source of light, preferably in a darkroom. It is what textbooks sometimes vaguely call "iridescence.") These colonies turned out to be ordinary staphylococci, but since the color phenomenon persisted in subcultures, I started making extracts of them with various solvents, hoping to isolate the elusive coloring substance. I failed in this, and then it dawned upon me that the phenomenon might be physical and not chemical. I first thought of fluorescence and then suddenly realized that I was dealing with diffraction of light, the surface culture acting as a diffraction grating. I went back to the original broth culture and found that the shiny particles were powdered glass and had nothing to do with the phenomenon, having merely found their way into the broth through a mistake of a technical assistant. (We had been using powdered glass for estimations of atmospheric bacteria, following a method then in vogue.) In pursuance of the idea that diffraction was the cause of the phenomenon, an apparatus was constructed in which a beam of white light was sent through a surface culture of staphylococci, and the resulting diffraction colors were collected and focused onto a screen.<sup>19</sup> A formula was developed enabling one to calculate the diameter of the cocci from the diameter of the diffraction spectra. By substituting a culture of round monilias for the cocci, a check was obtained on the correctness of the formula.<sup>19</sup> I then considered that the principle of diffraction, which, should also work with round  
lls.<sup>20-22</sup> It is shown in Fig. 1

how a beam of white light, sent through thin end of a blood film, undergoes diffraction at the edges of the red cells and produces sets of colored rings, arranged in rainbow or spectrum fashion and sometimes called halos. This is the origin of all diffraction methods for measuring red blood cells now in use.\*

It is shown in Fig. 1, and similarly in Figs. 2 and 3, that there is only one "complete" spectrum where all the colors are easily discernible. This is the spectrum to be used for measurements and comparison.

In the following years little scientific notice was taken of this method, except by Bergansius,<sup>2</sup> Millar,<sup>18</sup> Ponder,<sup>35, 36</sup> and Allen and Ponder.<sup>1</sup> During this time the technique was improved and applied to the measurement of various bacteria.<sup>24-26, 28</sup> It was not until 1924 that, stimulated by a publication of Hurst<sup>13</sup> on anemias, I applied the method to the differential diagnosis of anemias<sup>23</sup> and to their follow-up.<sup>29</sup> Various authors and firms then produced a variety of instruments for applying the method. Emmons<sup>7</sup> brought out the clinical eriometer. Eve<sup>8</sup> built a halometer which became strangely popular and which was followed by the direct halometer to which a Dr. Fine's name was attached. Pryce<sup>42</sup> simplified the halo method; Edwards<sup>6</sup> built a simple box of his own; Menzies<sup>17</sup> used a new refractometer (sic) designed by Dr. Malloy; Renshaw and associates<sup>43</sup> spoke of a hemovisor they were planning; and Bock<sup>4</sup> introduced the erythrocytometer which became widely used on the continent of Europe. Fullerton and co-workers<sup>10</sup> used an apparatus of their own and also a Ewles halometer. Christophers and Craighead<sup>5</sup> suggested an ingenious micromethod which employed a microscope, and Falisi<sup>16</sup> on similar lines produced a micro-diffractometer that was followed again by Smith<sup>15</sup> who introduced a diffractometer manufactured by the Spencer Lens Company which appears to work well, although in common with all the methods and apparatuses mentioned, it does not allow direct comparison of two blood films. Schalm<sup>11</sup> had a pocket apparatus built by Zeiss; Hogben<sup>14</sup> constructed an apparatus of his own; and finally there is Haden's<sup>13</sup> Haden-Hausser erythrocytometer. This list is probably not complete; it is likely that there are further variations of the same theme still unknown to me.

An improvement in technique came in 1929 when comparison was made in one apparatus of the diffraction patterns of two different blood films.<sup>30, 31</sup> This was done by cutting off one half of the set of colored circles produced by each of the two films and making the remaining semicircles share the same middle-line on the receiving screen. The arrangement in diagrammatic fashion is shown in Fig. 2. This allows direct comparison of an "abnormal" with a "normal" blood film, thus doing away with the need for actual measurement in many cases, simple inspection of the two diffraction patterns being sufficient to get the required information. This design also provides for direct comparison of two blood films taken from the same patient at different times, a very useful feature in the follow-up of patients. An apparatus on these lines can easily be put together by a technical assistant. Eventually this improved technique was em-

\*It was later brought to my notice that Young<sup>30</sup> in 1813 applied the same principle to the measurement of wool fibers (eriometer) and later of red blood cells. His work was never followed up.



FIG. 1.

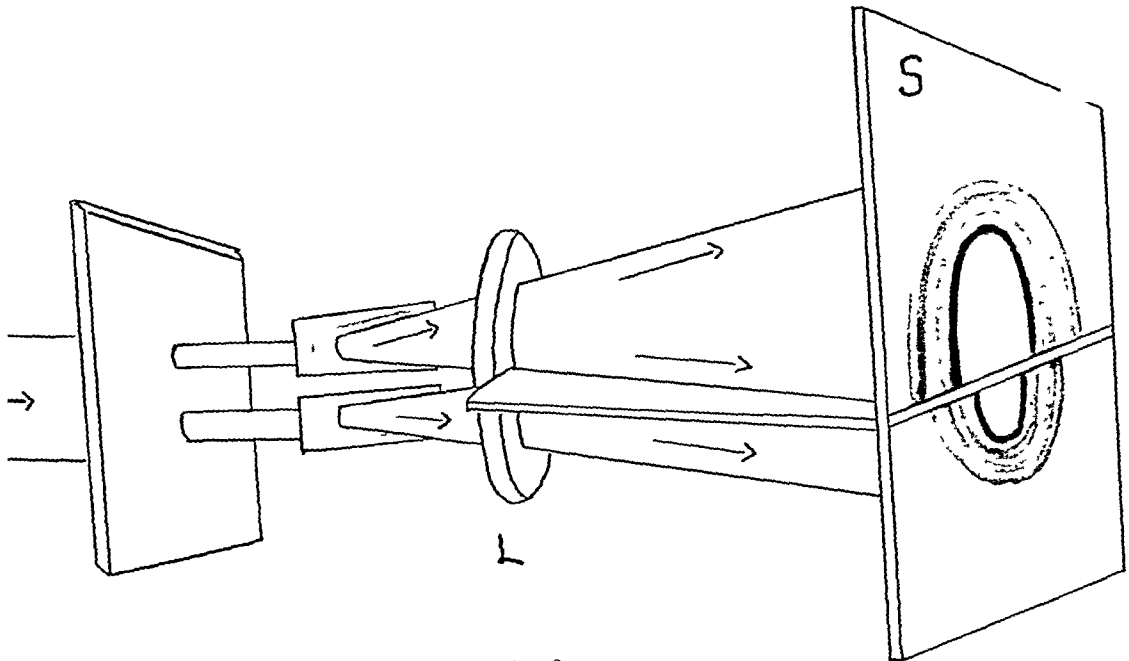
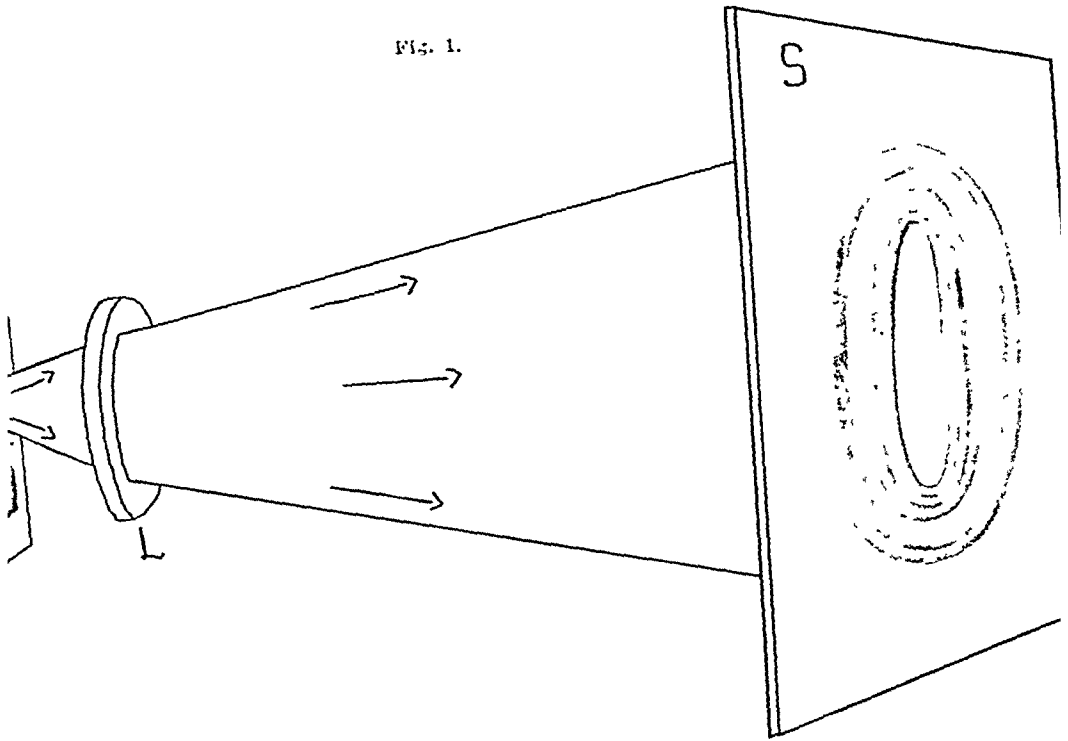


FIG. 2.

Fig. 1.—Diagram of blood film serving as diffraction grating.  
Fig. 2.—Diagram of apparatus, known as Pijper's Blood Cell Tester. Direct comparison of two blood films, normal or standard blood film on one side, pernicious anemia on the other.



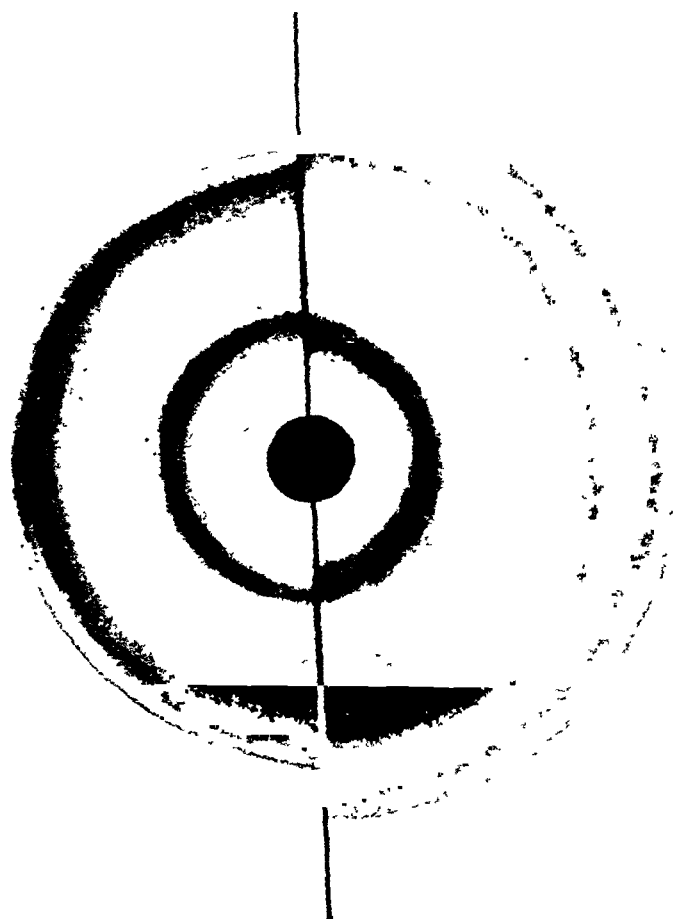


Fig. 3.—What appears on screen of apparatus when normal or standard blood is compared with anemia caused by prolonged hemorrhages, malignant growth, or similar conditions. Spectrum on right is broader, but yellow circle is not displaced. This means normal mean diameter with increased anisocytosis.



bodied in the apparatus placed on the market in 1935 by Zeiss as Pijper's Blood Cell Tester.<sup>13-14</sup> Its shape and size are the result of cooperation between Prof. Siedentopf, technical adviser to the Zeiss firm, and myself. It is based on the idea illustrated in Fig. 2. Pictures of it can be found in some textbooks.<sup>15</sup> In Fig. 2 the spectra produced by a normal blood film and a blood film from a case of pernicious anemia are compared. In Fig. 3 is shown what actually appears on the ground-glass screen of the cell tester when normal blood and blood from a case of anemia caused by prolonged hemorrhage, malignant growth, or similar conditions are compared.

#### PURPOSE OF PAPER

The purpose of this paper is to show that, contrary to what is usually stated in textbooks and other publications, the diffraction method of measuring red cells, if properly applied, is capable not only of determining the mean diameter of about one million cells, but also of determining the degree and quality of anisocytosis present in a blood film. A good diffraction apparatus should allow three measurements or determinations. One gives the mean diameter, the second gives the diameter of the smallest cells present in appreciable numbers, and the third does the same for the largest cells. All this can be done in a few minutes and in this way degree and quality of anisocytosis are determined. I do not know of any other apparatus that is capable of doing this. The technical side of this paper therefore applies to the apparatus known as Pijper's Blood Cell Tester or to any apparatus built on identical lines.<sup>16</sup>

#### IMPORTANCE OF DETERMINING RED CELL DIAMETERS

Modern hematology emphasizes morphology. Importance is attached to shape and size of red blood cells. Hematology owes a good deal to the careful microscopic measurements of red cells by Price-Jones and associates.<sup>17-21</sup> This work brought revelations, but apart from an occasional special case, Price-Jones' method and its substitutes are too elaborate for daily application.

The old-fashioned determination of the hemoglobin index as an indication of the size of the red cell still lingers on. This is indefensible because its value is arrived at by dividing a hemoglobin value by a red cell value, neither of which is usually determined with sufficient accuracy. It is admitted that by using a photoelectric colorimeter, errors in hemoglobin estimations can be very much reduced, but there is no similar reliable method yet for red cell counts. It is known that in ordinary work, with the apparatus available in most laboratories, an error of 5 per cent in both these determinations is unavoidable, an error of 10 per cent is quite common, and higher errors occur. A calculation based on two incorrect values should in principle be avoided as it leads to still more erroneous final results. What may happen, unless particular care is taken, is illustrated in Table I.

\*At the present time, as far as I know, no blood cell testers can be obtained from the Zeiss firm. Perhaps an American firm will find it worth while to build an apparatus on the same lines and of identical dimensions, so that the numerical data supplied in this paper will apply.

TABLE I. ERRORS IN DETERMINATION OF HEMOGLOBIN COLOR INDEX

	HEMOGLOBIN (PER CENT)	RED CELLS	INDEX
Supposed real values	90.0	4.0	1.1
Errors of 5 per cent	85.5	4.2	1.0
Errors of 5 per cent	94.5	3.8	1.2
Errors of 10 per cent	81.0	4.4	0.9
Errors of 10 per cent	99.0	3.6	1.4

It is seen that through errors often accompanying the method, an index which in reality is 1.1 can appear as high as 1.4 or as low as 0.9. Index determinations therefore are inadvisable.

Similar objections can be raised against determinations of mean red cell volume by dividing hematocrit values by number of cells. What may happen, unless particular care is taken, is illustrated in Table II.

TABLE II. ERRORS IN DETERMINATION OF MEAN CELL VOLUME

	VOLUME OF PACKED CELLS (PER CENT)	NUMBER OF RED CELLS	MEAN CELL VOLUME
Supposed real values	39.2	3.80	103
Errors of 5 per cent	41.1	3.61	114
Errors of 5 per cent	37.3	3.99	93
Errors of 10 per cent	43.1	3.42	126
Errors of 10 per cent	35.3	4.18	84

It is seen that in a case where the mean red cell volume in reality is 103, errors often accompanying the method may lead to erroneous determinations as high as 126 or as low as 84.

There is, of course, nothing new in this criticism; many authors admit the shortcomings of these methods (Todd and Sanford<sup>46</sup> and Wintrobe<sup>49</sup>). In experienced hands the method for determination of mean red cell volume still works reasonably well, but many workers do not seem to be conscious of the fundamental fact that it is unsatisfactory to try and arrive at a correct value by dividing two incorrect values one by the other. The problem confronting hematologists nowadays is not only to place obvious cases into the correct category, but to deal with and to follow up early and mild cases in a satisfactory manner. These are the cases where deviation from the normal may be minimal, and in such cases involved calculations on not very accurate data may become misleading. There are, for example, cases of early subacute-combined degeneration of the cord, and cases of mental changes due to early pernicious anemia as described by Warburg and Jørgensen,<sup>47, 48</sup> where hemoglobin and red cells show too little change to lead to reliable results in the determination of either hemoglobin index or mean cell volume. There, in my experience, a direct determination of red cell diameter by the diffraction method may definitely show a quick way to the correct diagnosis.

#### THEORY OF DIFFRACTION METHOD

Even professional physicists admit great difficulties in attempts to give a full explanation as to what happens when a beam of light passes through a blood

film. The following formula, which applies to the conditions of Figs. 1 and 2, has, however, found favor among physicists and answers well in practice.

$$\text{Diameter red cells} = \frac{1.7 \lambda \sqrt{f^2 + r^2}}{r}$$

In this formula " $\lambda$ " is the wave length of light under consideration, " $f$ " is the focal distance of the lens ( $L$ ) in Figs. 1 and 2 which focuses the diffraction pattern on the screen ( $S$ ), and " $r$ " is the radius of the colored circle of wave length " $\lambda$ ."

In the blood cell tester the lens has a focal distance of 20 cm., and " $r$ " varies between 1.5 to 3.5 cm. As " $r$ " is so much smaller than " $f$ ," the formula can be simplified by eliminating " $r^2$ ." A simple calculation will show that the average error introduced thereby is not more than 1 per cent, which is within the experimental error of the method. The formula thus becomes:

$$\text{Diameter red cells} = \frac{340,000 \lambda}{r}$$

The colored circles which are of importance are those of yellow, outer edge of red, and innermost violet. This means one color rather near the middle of the spectrum and two others at the extreme ends of the spectrum. Yellow is chosen because it is a narrow band, and therefore easy to read, and quite near enough the middle of the spectrum to be suitable.

The position would be very simple if all red blood cells in a given film were of identical diameter. This is not the case in normal blood, and less so in abnormal blood. Price-Jones<sup>37-40</sup> has shown that red blood cells form a population and their size distribution follows a frequency curve. There are kinds of anemia where this curve is a good deal flatter than normal, in other words where the only abnormality is an increased degree of anisocytosis. Normal blood has cells of a mean diameter of about 7.5 microns, but through normal anisocytosis there is an ever-decreasing number of smaller and larger cells.

If all red blood cells in a given film were of identical diameter, it would not matter which color was chosen for applying the formula, the result would be the same in any case. The position which arises from the ever-present anisocytosis is illustrated in Fig. 4.

Shown in Fig. 4 in the left top quadrant are the three main colored circles resulting from a normal blood film as produced by diffraction by the cells of 7.5 microns, which are the most numerous ones and therefore produce the brightest colors. In a normal film the anisocytotic spread will comprise cells from 7.5 to about 6.7 microns, on the one side, and cells from 7.5 to about 8.5 microns, on the other side. In the bottom left quadrant of Fig. 4 are shown the colors produced by the largest cells, and in the top right quadrant the colors produced by the smallest cells. In actual practice of course the three sets of colored rings are superimposed, giving rise to the bottom right quadrant.

It is seen that the final position of the yellow ring is hardly affected by the superimposition of the diffraction patterns of all the red cells. It therefore

can safely be used for the calculation by means of the formula of the mean diameter of all the red cells.

It is also obvious that in the final spectrum the outer edge of the red circle is the expression of the diameter of the smallest cells in the film, and the innermost violet circle the expression of the diameter of the largest cells. These colored circles thus can be safely used in the formula for the calculation of the smallest and largest cells present. Application of the formula three times, once for yellow, once for red, and once for violet, gives the mean diameter and the smallest and largest cells, in other words, the kind and degree of anisocytosis present.

It should now be clear that any diffraction method which bases its measurements on anything but the yellow circle for the calculation of mean diameter is faulty. A measurement based on the position of the outer edge of the red circle, as is often done, will result in calculating not the mean diameter, but the diameter of the smallest cells. In normal blood this may not make much difference, as the values for mean diameter and smallest diameter lie close together. But in cases where anisocytosis is increased, and there are cases where increased anisocytosis is the main or even only abnormal feature, a serious error will be committed. Increased anisocytosis means a broader total spectrum, the distance between the outer edge of the red ring and the innermost violet ring being larger than normal. It is one of the advantages of the cell tester that such an increase in anisocytosis can be read at a glance, through the direct comparison with normal blood (Fig. 3).

#### TECHNIQUE OF READING RESULTS AND MEASURING

The essence of good work with blood films for any purpose is even distribution of cellular elements over a glass surface. Measurements, whether by microscopic methods or by diffraction, can only be reliable if the red cells lie separate over a large area. Any diffraction method needs a circular area of this kind of at least a quarter of an inch in diameter. If there are many cells lying partly on top of one another, one does not measure the diameter of single cells, and the results will be vitiated. A properly made blood film, made by pushing the polished narrow edge of a microscope slide along the surface of another microscope slide, with a drop of blood inside the angle made by the two slides, shows a silky glossy area at the end of the film. The speed of the movement and the size of the angle determine the quality of the film; practice is essential to acquire the art. It is only the silky area at the end of the film that can be used for diffraction measurements (Fig. 5). A simple way of checking on the suitability of a blood film is to look through its thin silky end at a distant lamp. One should then see a number of bright rainbows round the lamp. It is only with pronounced poikilocytosis that the rainbows are not so bright.

The film is moved about on the stage of the apparatus over the little hole which admits the light until one has found the spot which produces the brightest and clearest colors. This is a matter of experience.

On the screen of a properly designed apparatus for the diffraction measurement of red blood cells, such as the blood cell tester, one sees two sets of con-



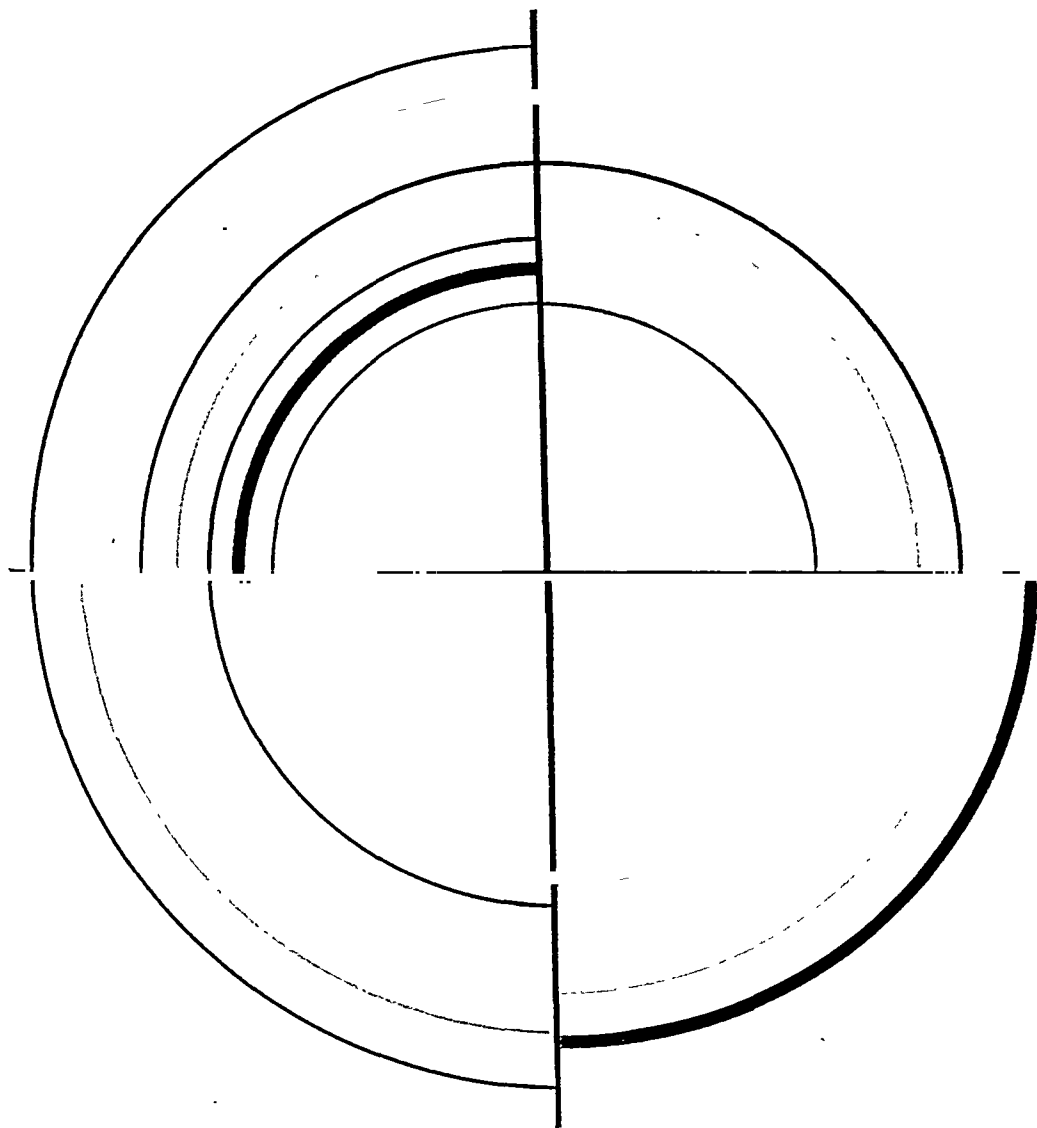


Fig. 4.—Superposition of spectral rings produced by the varying diameters of the red blood cells in a normal film.



centric colored circles arranged in rainbow fashion, as in Fig. 3. Starting from the center, where a bright patch of white light is covered by a small black shield, and traveling to the periphery, one first goes through a reddish-brown circle. Then follows the violet of the spectrum that is used for measurement and comparison. The further colors of the rainbow appear in orderly fashion, blue, green, yellow, orange, and red. The red circle is surrounded by a green circle, which belongs to the next spectrum and which for reasons into which I need not go now is somewhat confused. After this green there may be another red circle, followed again by green, and so on. For purposes of measurement and comparison one need only to take notice of the complete spectrum ranging from the innermost violet to the outer edge of red. In Fig. 3 it is at once obvious that the total spectrum on the right-hand side is a good deal wider than the one on the left. The one on the left is produced by a normal blood film and serves as a standard. There are minor individual variations in what is usually regarded as normal blood, but as a rule a properly prepared blood film made from a healthy person with a hemoglobin content of over 15 Gm. per 100 ml. will serve.



Fig. 5.—Properly made blood film, the thin silky area at the end to be used.

Such a standard blood film, unfixed and unstained, will remain serviceable for many months. In Fig. 3 it is further noticed that the yellow circle on the right side, produced by the blood under examination, is of about the same width as that of the standard blood on the left. The yellow circle being the expression of the mean diameter of the red blood cells, it becomes obvious that the mean diameter of the red blood cells under examination can be regarded as normal. If absolute values for this mean diameter are desired, a pair of compasses is used, placing the sharp point of one of the legs on the yellow circle where it touches the black line dividing the two sets of colored circles, and then the sharp point of the other leg across the center on the opposite section of the yellow circle. The distance between the two ends of the compasses is then measured on a millimeter rule and the value found converted into microns by means of the conversion table (Table III) which is based on the formula previously given. For the mean diameter one uses the column in Table III marked Yellow. If, for example, the value found in millimeters is 54, this corresponds in the conversion table to a value of 7.5 microns for the mean diameter. With practice this procedure can be performed with an accuracy of 1 mm., which corresponds to 0.1 or 0.2 microns.

The extreme edge of the red circle is the expression of the diameter of the smallest cells present in appreciable numbers in the blood film. In Fig. 3 the spectrum on the right-hand side shows that this edge extends much farther outward than with the normal blood on the left-hand side. This means that there is increased anisocytosis in the blood under examination, and this is confirmed by the innermost violet circle which is the expression of the diameter of the largest cells present in appreciable numbers and which is situated much farther inwards than in the case of the normal blood. For absolute measurements one proceeds as follows. For the extreme edge of the red circle place the sharp points of the compasses where the green circle which surrounds the extreme edge of the red one just begins, so as to make sure that the extreme edge of the red circle where the red color is faint is really taken into account. For the innermost violet, measure as near the brownish-red inner circle as possible. The millimeter values found are transferred to a millimeter rule and converted into microns by means of the conversion table (Table III), using the appropriate columns for Red and Violet. Here, too, the error should not exceed 1 mm., which corresponds to an accuracy of 0.1 or 0.2 micron. In extreme cases the error becomes larger, but also proportionally less important.

TABLE III. CONVERSION TABLE

RING IN MILLIMETERS	CELL DIAMETERS IN MICRONS			RING IN MILLI- METERS	CELL DIAMETERS IN MICRONS		
	YELLOW	VIOLET	RED		YELLOW	VIOLET	RED
25	16.5	11.7	18.3	54	7.5	5.3	8.3
26	15.8	11.2	17.6	55	7.3	5.2	8.1
27	15.2	10.8	16.9	56	7.2	5.1	8.0
28	14.6	10.4	16.3	57	7.1	5.0	7.9
29	14.1	10.0	15.7	58	6.9	4.9	7.7
30	13.6	9.7	15.2	59	6.8	4.8	7.6
31	13.2	9.3	14.7	60	6.7	4.7	7.5
32	12.8	9.0	14.2	61	6.6	4.7	7.3
33	12.3	8.7	13.8	62	6.5	4.6	7.2
34	12.0	8.5	13.3	63	6.4	4.5	7.1
35	11.6	8.2	12.9	64	6.3	4.4	7.0
36	11.3	8.0	12.6	65	6.2	4.4	6.9
37	11.0	7.8	12.2	66	6.1	4.3	6.8
38	10.7	7.6	11.9	67	6.0	4.2	6.7
39	10.4	7.4	11.6	68	5.9	4.2	6.6
40	10.1	7.2	11.3	69	5.8	4.1	6.5
41	9.9	7.0	11.0	70	5.7	4.1	6.4
42	9.6	6.8	10.7	71	5.6	4.0	6.3
43	9.4	6.7	10.5	72	5.6	3.9	6.2
44	9.2	6.5	10.2	73	5.5	3.9	6.1
45	9.0	6.4	10.0	74	5.4	3.8	6.0
46	8.8	6.2	9.8	75	5.3	3.8	5.9
47	8.6	6.1	9.6	76	5.3	3.7	5.9
48	8.4	6.0	9.4	77	5.2	3.7	5.8
49	8.2	5.8	9.2	78	5.1	3.6	5.7
50	8.1	5.7	9.0	79	5.1	3.6	5.6
51	7.9	5.6	8.8	80	5.0	3.5	5.6
52	7.7	5.5	8.6	81	4.9	3.5	5.5
53	7.6	5.4	8.5				

Conversion table to be used in connection with Pijper's Blood Cell Tester, or any other which uses a lens of 20 cm. focal distance. The diameters of the colored st and complete spectrum are given in millimeters, and from these the found in the table. Thus, a yellow ring of 54 mm. would mean a mean microns; a red ring of 64 mm. would mean that the smallest cells present in appreciable numbers would measure 7.0 microns; and a violet ring of 36 mm. would mean that the largest cells present in appreciable numbers would measure 8.0 microns.

The directions for reading and measuring and the conversion table (Table III) given here are based on theoretical considerations, amplified by long experience and supported by actual comparison of results obtained by direct microscopic methods and the diffraction method.

#### CLINICAL APPLICATIONS OF DIFFRACTION METHOD AND COMPARISON WITH MICROSCOPIC MEASUREMENTS

A comparison of the results of direct microscopic measurements with the results of the diffraction method applied to a small number of cases has been published.<sup>32</sup> It was found that the results corresponded quite well. Similar work, but in less detail, has been undertaken with essentially the same results by Bernstein,<sup>3</sup> Smith,<sup>45</sup> Knoche,<sup>18</sup> and especially Haden.<sup>11, 12</sup>

I have now collected a large number of cases with two sets of measurements undertaken on the same blood film, one by the diffraction method as detailed in this paper and one by fixing and staining the cells and then measuring them with the filar micrometer eyepiece of Bausch and Lomb. With the microscopic method 500 cells were measured in every case, taking the cells as they came, avoiding any temptation toward selection. The results of the microscopic measurements were then plotted in the form of a Price-Jones curve. These are given in Figs. 6 to 48, and underneath each curve the results of the diffraction measurements are given by three crosses. The middle cross indicates the mean diameter, and the other two indicate the diameters of the largest and smallest cells present in appreciable numbers. From the position of the crosses one can read off the degree and kind of anisocytosis present.

It cannot be expected that complete correspondence will be reached in all cases. The shape of a Price-Jones curve is the expression not only of variation in cell diameter, but also of variation in error committed in the computation of cell diameter. In addition, there is the "personal equation." It is my experience that no two technical assistants will return the same curve from one and the same blood film. The personal equation counts for much less with the diffraction method if it is performed as described herein, because there always is the direct comparison with normal blood. It follows from the nature of the diffraction method that the very smallest and the very largest cells are lost sight of, their number being too small to produce an appreciable amount of color. On the other hand, the diffraction method deals with at least a million cells, as against a few hundred dealt with by microscopic measurements, and with the diffraction method "selection" is excluded.

The diffraction method requires experience and good color vision, especially where through extensive anisocytosis the colors are spread over a larger area and become somewhat thin. Microscopic measurements, however, demand similar qualities. Checking results is easy with the diffraction method because it works so quickly, but it is hardly feasible with microscopic measurements without wasting an enormous amount of time. Marked poikilocytosis naturally causes watery spectra, somewhat difficult to measure, but then there always is the direct comparison with normal blood. Nobody who has tried it can be very

satisfied with microscopic measurements in such a case. A case of pronounced pernicious anemia may through poikilocytosis give an initial blood film with a very small spectrum and poorly defined colors, but this as such already represents useful information. It becomes even more useful if after a week's treatment, the colors, in films prepared then, become more defined; if the two films are then placed side by side in the apparatus, even minor improvements in poikilocytosis can be read at a glance. One can repeatedly collect films from such a patient and directly compare them in succession with one another in the apparatus and so get a complete picture of the progress of the case.

Figs. 6 to 48 refer to a selection of cases in which red cell measurements were undertaken on blood films, both by the diffraction method and by microscopic measurement.

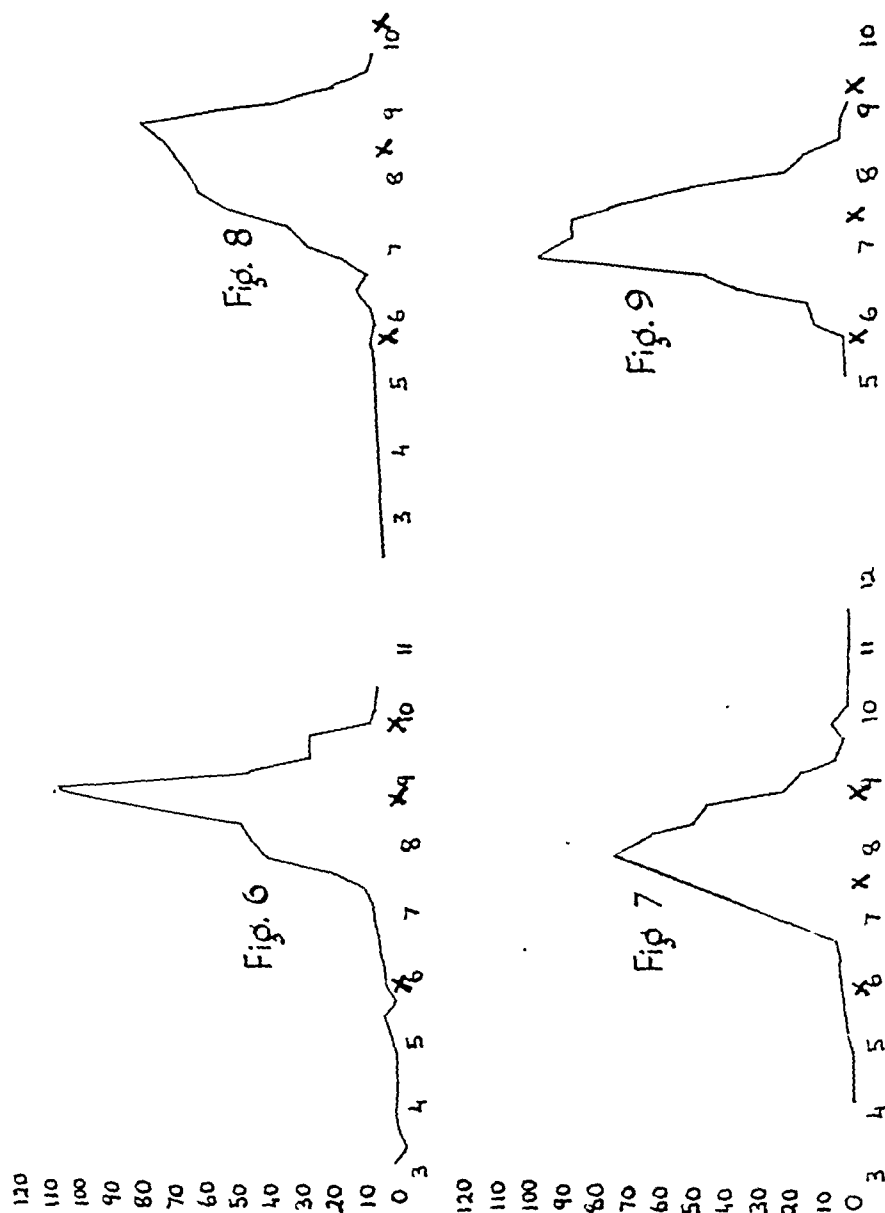
Figs. 49 and 50 illustrate how cases can be followed by means of the diffraction method. Each horizontal line gives the cell diameters of a patient on a particular day, the three crosses representing, respectively, the mean diameter (middle cross) and the smallest and largest cells (the other two crosses). The values can be read off in microns. The first 8 lines marked PA 1 refer to a patient with pernicious anemia and give the cell diameters in consecutive weeks, the first line indicating the position before treatment started, the second line after one week's treatment, and so on, until after seven weeks more or less normal conditions are reached. The difference after one week was already striking, and the improvement continued every week. The larger cells showed a more rapid return to normal than did the mean diameter, and the mean diameter again changed more quickly and markedly than the smallest cells.

The following five lines, marked PA 2, in similar fashion refer to another patient with pernicious anemia, except that the first interval here was only four days, the other intervals again being a full week. Obvious improvement took place within the short time of four days' treatment, which was continued during the further weeks. Examinations stopped before full restoration was reached.

The next seven lines, marked PA 3, refer to a patient with pernicious anemia who was examined every week. Improvement was quite definite after one week, and more so after a fortnight, but then the patient neglected treatment for two weeks; this is reflected in the cell diameters, the cells increasing again in size, with anisocytosis becoming more marked. Treatment was instituted again, and improvement in cell diameters was again achieved, but the patient responded poorly and progress was not satisfactory, as shown by the cell diameters.

The lines and crosses marked PA 4, PA 5, PA 6, PA 7, PA 8, and PA 9 all refer to patients with pernicious anemia who were not followed for comparably long periods; the intervals between the separate individual observations varied between a fortnight and three weeks. Case PA 8 neglected treatment and this becomes manifest in the cell diameters.

These further observations confirm that at every control examination during progress it is always the largest cells that show the biggest relative improve-



Figs. 6-13.—Pryce-Jones curves of various blood films, with the results of diffraction measurements indicated by crosses underneath.

Fig. 6.—Old case of pernicious anemia; treatment recently neglected. Hemoglobin, 11.6 Gm.; red cells, 1.6 million. Correspondence between two methods of measurement quite good. Just as in several of the following figures, it is noticeable that the diffraction method missed the very small and the very large cells which were present in very small numbers anyhow.

Fig. 7.—Same case as in Fig. 6, but now had had good treatment for four months. Hemoglobin, 14.5 Gm.; red cells, 5.2 million. Correspondence between two methods not so good, but "shift to left" and decrease in anisocytosis obvious by both methods.

Fig. 8.—Triturated case of pernicious anemia. Hemoglobin, 8.2 Gm.; red cells, 2.9 million. Correspondence quite good.

Fig. 9.—Same case as in Fig. 8; had now had treatment for two months. Hemoglobin, 13.1 Gm.; red cells, 5.5 million. Correspondence good; anisocytosis more pronounced with diffraction method than with microscope method.

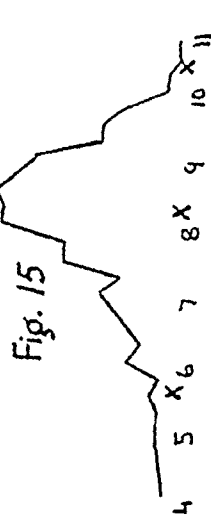
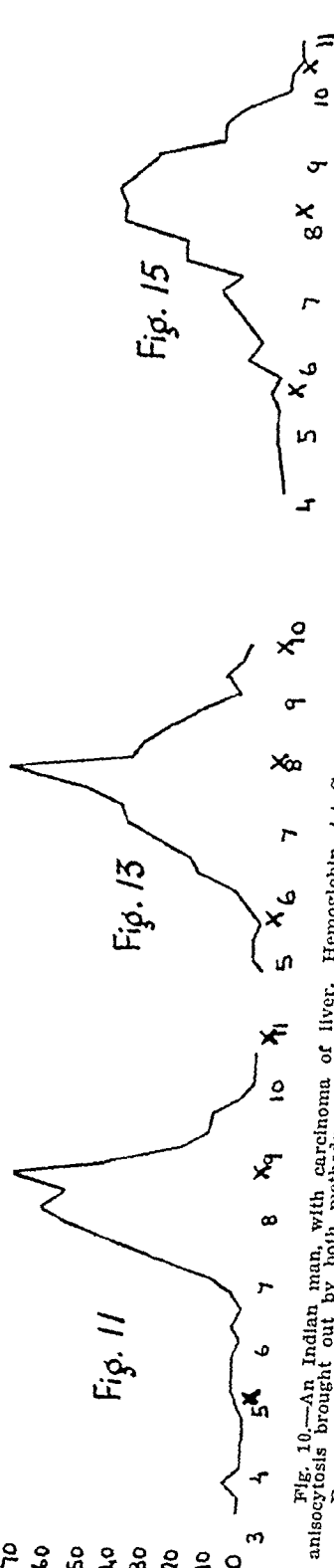
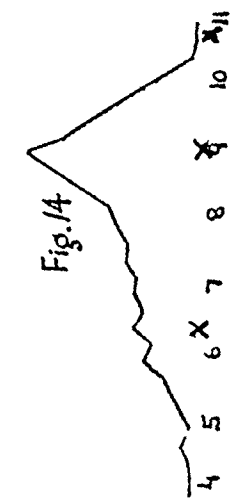
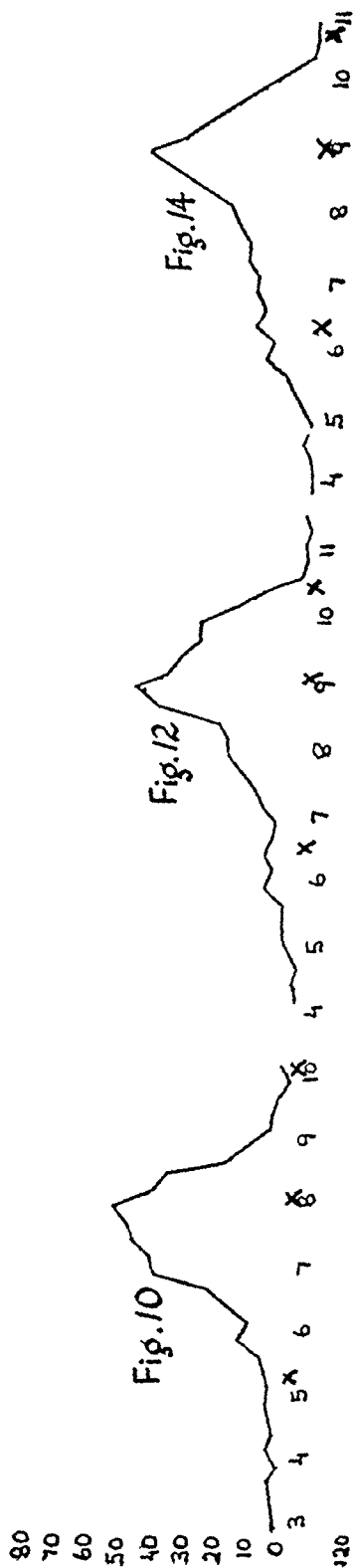


Fig. 10.—An Indian man, with carcinoma of liver. Hemoglobin, 4.4 Gm.; red cells, 1.3 million. Note "shift to right" and very marked anisocytosis brought out by both methods.

Fig. 11.—Pernicious anemia, untreated. Hemoglobin, 6.1 Gm.; red cells, 1.6 million. Both methods showed very similar conditions of red cells; the diffraction method showed more anisocytosis.

Fig. 12.—Pernicious anemia, untreated. Hemoglobin, 4.4 Gm.; red cells, 2.0 million. Correspondence quite good, except for some more smaller cells showing up by microscopic method.

Fig. 13.—Same case as in Fig. 12, but now had had several treatments for several weeks. Hemoglobin, 9.9 Gm.; red cells, 3.7 million. Note "shift to left," but cells still remained large and anisocytosis very marked.

Fig. 14.—Pernicious anemia. Hemoglobin, 6.7 Gm.; red cells, 2.8 million. Correspondence good except for some smaller cells, which had identical "shift to left" coming to light by both methods. Cells with same "shift to left" as in Fig. 14, but now had had treatment for two weeks. Hemoglobin, 9.7 Gm.; red cells, 3.7 million.

Fig. 15.—Same case as in Fig. 14, but now had had treatment for two weeks. Hemoglobin, 9.7 Gm.; red cells, 3.7 million. Note "shift to left" coming to light by both methods. Cells with same "shift to left" as in Fig. 14, but now had had treatment for two weeks. Hemoglobin, 9.7 Gm.; red cells, 3.7 million.



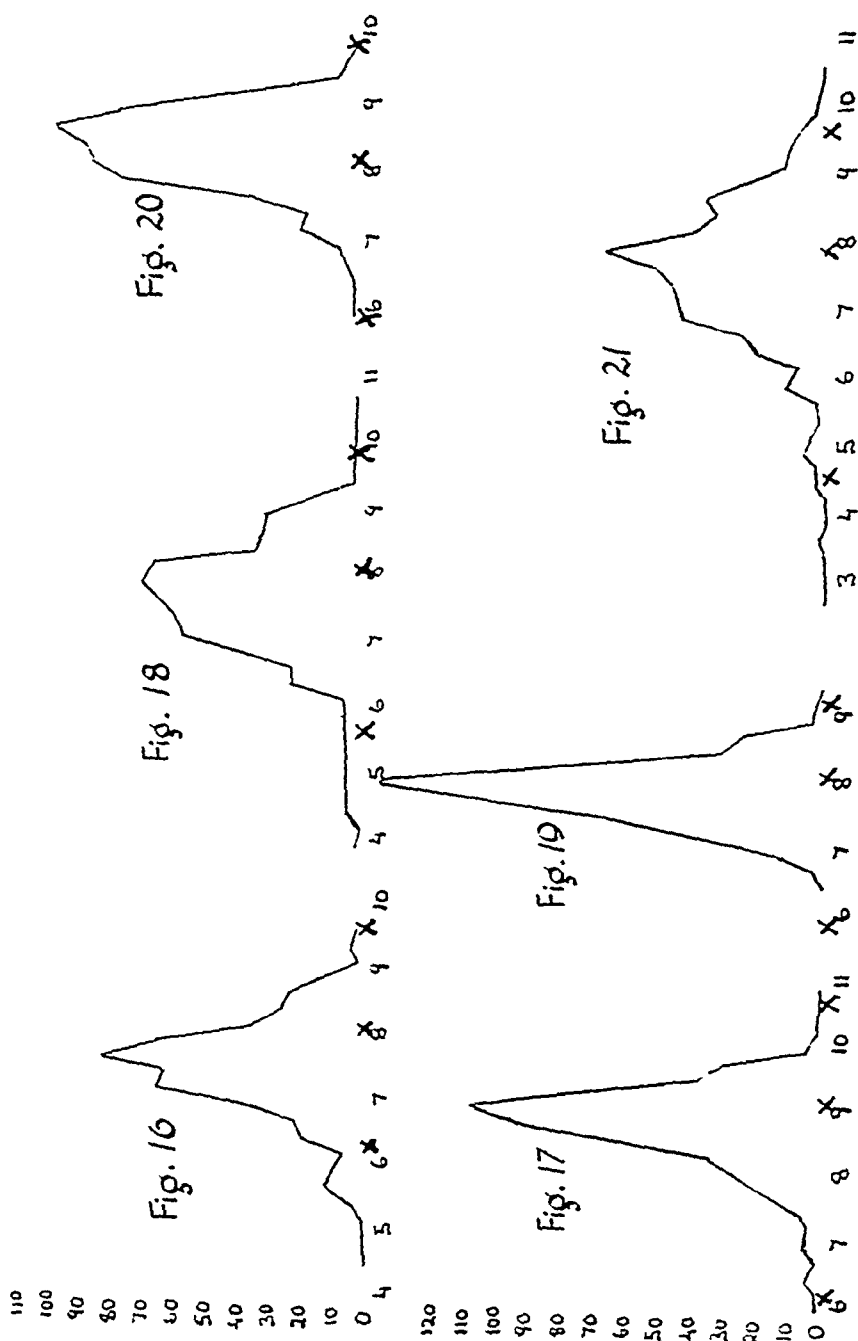


Fig. 16.—Same case as in Figs. 11 and 15, but now had had six weeks of treatment. Hemoglobin, 11 Gm.; red cells, 5.1 million. Cells still rather too large and too much anisocytosis, both abnormalities appearing more pronounced with diffraction method.

Fig. 17.—Pericious anemia; had had irregular treatment. Hemoglobin, 12.6 Gm.; red cells, 1.7 million. Cells still much too large, anisocytosis more than normal, both changes more obvious with diffraction method.

Fig. 18.—Insufficiently treated pernicious anemia. Hemoglobin, 5.1 Gm.; red cells, 2.6 million. Note marked anisocytosis and large cells, as shown by both methods.

Fig. 19.—Subacute combined degeneration of spinal cord. Hemoglobin, 13.9 Gm.; red cells, 5.7 million. Note large cells with hardly more anisocytosis than normal. This is the kind of case that is quickly diagnosed by diffraction method.

Fig. 20.—Obsolete jaundice in child. Hemoglobin, 14.3 Gm.; red cells, 5.3 million. Cells large, with marked anisocytosis, rather similar picture by both methods.

Fig. 21.—Pernicious anemia. Hemoglobin, 5.9 Gm.; red cells, 2.0 million. Correspondence very good.

Fig. 22

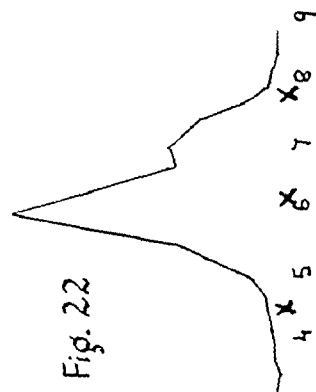


Fig. 24

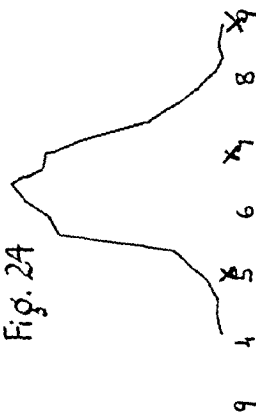


Fig. 26

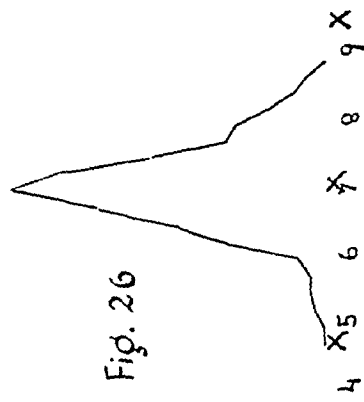


Fig. 23

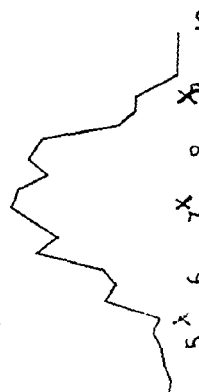


Fig. 25

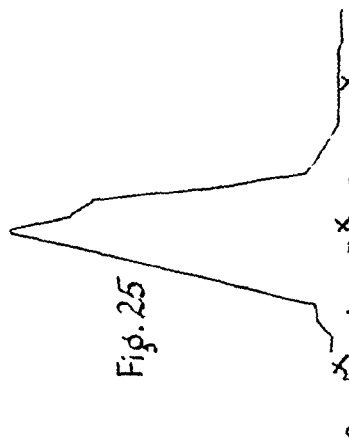


Fig. 27

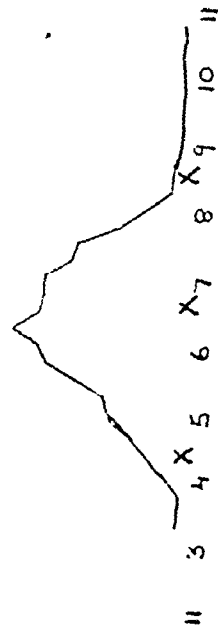


Fig. 22—Famulal haemolytic jaundice. Hemoglobin, 6.9 Gm.; red cells, 3.5 million. Fragility of red cells; hemolysis started at 6.5 per cent, was complete at 0.38 per cent. Spleen taken out next day.

Fig. 23—Same case as in Fig. 22, spleen taken out two weeks before. Note especially 'smallness' of cells, which suggested diagnosis in diffraction apparatus whole micron to the right, but anisocytosis much the same. Hemoglobin, 7.9 Gm.; red cells, 5.7 million. All cells shifted about a micron to the right.

Fig. 24—Carcinoma of colon. Hemoglobin, 4.1 Gm.; red cells, 3.6 million. Cells rather small, anisocytosis increased, picture was difficult to interpret, but excluded pernicious anemia and urged examiner to look for other causes.

Fig. 25—Prolonged bleedings from hemorrhoids. Hemoglobin, 8.7 Gm.; red cells, 2.9 million. Mean diameter normal, but anisocytosis marked, much more so by diffraction method than by microscopic method.

Fig. 26—Prolonged uterine hemorrhages. Hemoglobin, 6.3 Gm.; red cells, 3.3 million. Note normal mean diameter and again marked anisocytosis.

Fig. 27—Prolonged uterine hemorrhages. Hemoglobin, 1.8 Gm.; red cells, 2.3 million. Mean diameter somewhat small by both methods, but main feature was extreme anisocytosis, also readily visible by both methods.

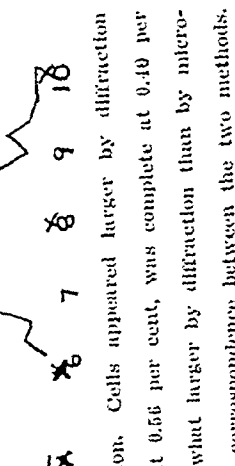
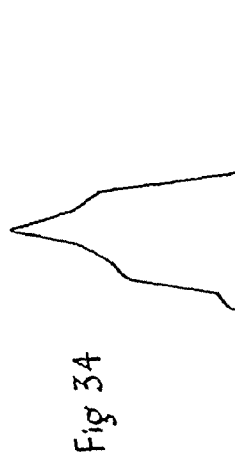
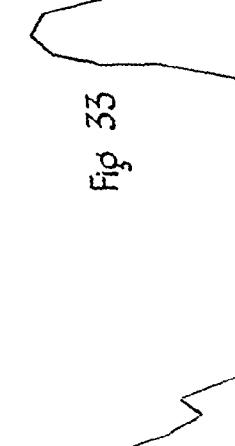
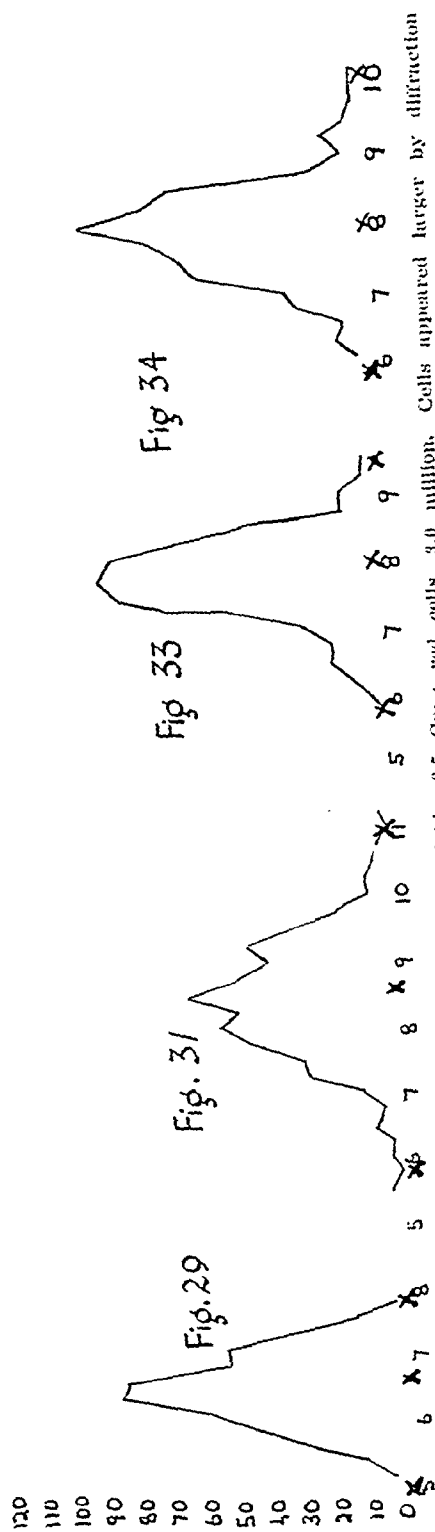
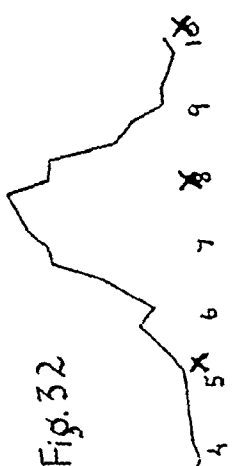
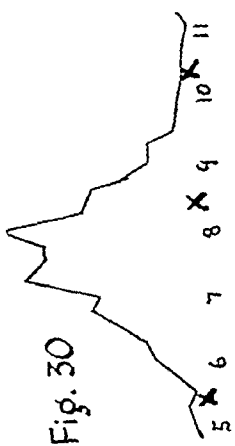
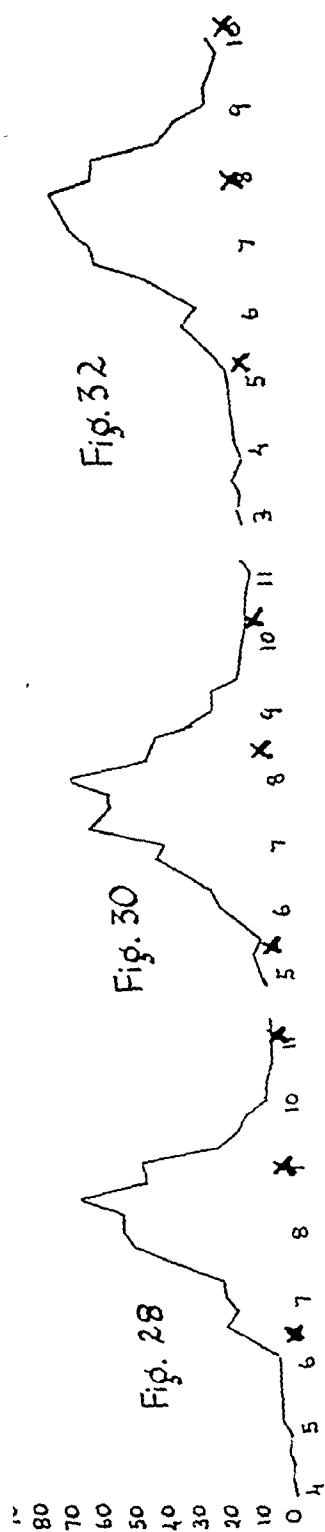


Fig. 28.—Rather typical case of pernicious anemia. Hemoglobin, 8.5 Gm.; red cells, 3.0 million. Cells appeared larger by diffraction method, but main features of both sets of data were identical.

Fig. 29.—Acetabular jaundice. Hemoglobin, 14.1 Gm.; red cells, 4.7 million. Fragility: started at 0.56 per cent, was complete at 0.40 per cent. Cells very small; anisocytosis increased. Mean diameter somewhat larger by diffraction than by microscope measurement.

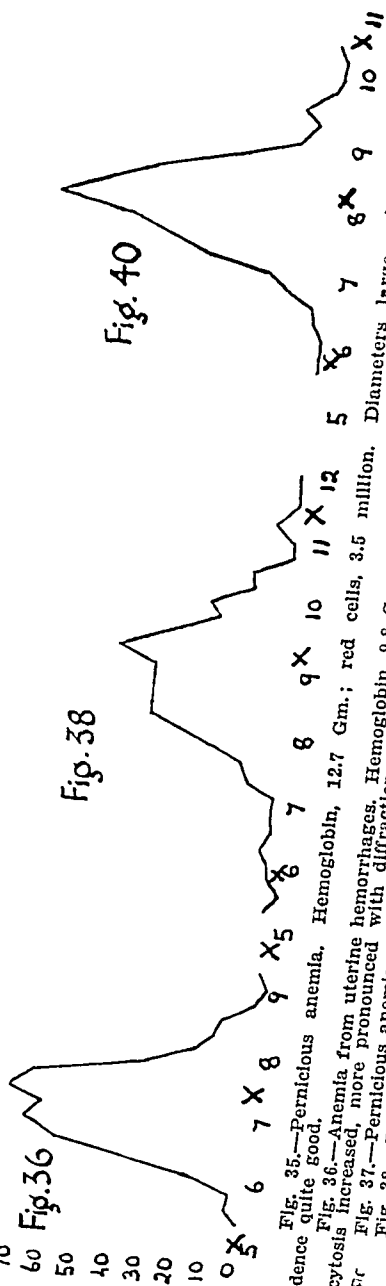
Fig. 30.—Pernicious anemia. Hemoglobin, 8.9 Gm.; red cells, 2.3 million. Cells large; excellent correspondence between the two methods.

Fig. 31.—Pernicious anemia. Hemoglobin, 12.4 Gm.; red cells, 3.9 million. Cells enlarged; correspondence quite good.

Fig. 32.—Liver carcinoma. Hemoglobin, 6.5 Gm.; red cells, 2.1 million. Cells still large and increased anisocytosis; identical by both methods.

Fig. 33.—Treated pernicious anemia. Hemoglobin, 15.4 Gm.; red cells, 4.9 million. Cells, however, still large and increased anisocytosis; identical by both methods.

Fig. 34.—Treated pernicious anemia. Hemoglobin, 15.4 Gm.; red cells, 4.9 million. Cells, however, still large and increased anisocytosis; identical by both methods.



dence quite good.

Fig. 36.—Anemia from uterine hemorrhages. Hemoglobin, 12.7 Gm.; red cells, 3.5 million. Diameters large, with increased anisocytosis; correspondence quite good.

Fig. 37.—Pernicious anemia. Hemoglobin, 9.8 Gm.; red cells, 3.1 million. Mean diameter large and same as normal; chief abnormality was anisocytosis which was increased, more so by the diffraction method than by microscopic measurement.

Fig. 38.—Pernicious anemia. Hemoglobin, 13.1 Gm.; red cells, 3.1 million. Cells enlarged, anisocytosis increased, correspondence quite good.

Fig. 39.—Anemia through prolonged mental illness, neglect, and starvation. Hemoglobin, 3.8 Gm.; red cells, 2.1 million. Mean diameter large and same as normal; chief abnormality was anisocytosis which was increased, more so by the diffraction method than by microscopic measurement.

Fig. 40.—Pernicious anemia; marked nervous symptoms. Hemoglobin, 11.1 Gm.; red cells, 2.6 million. Mean diameter large and same as normal; chief abnormality was anisocytosis which was increased, more so by the diffraction method than by microscopic measurement.

Fig. 41.—Pernicious anemia. Hemoglobin, 12.7 Gm.; red cells, 3.5 million. Diameters large, with increased anisocytosis; correspondence quite good.



Fig. 46

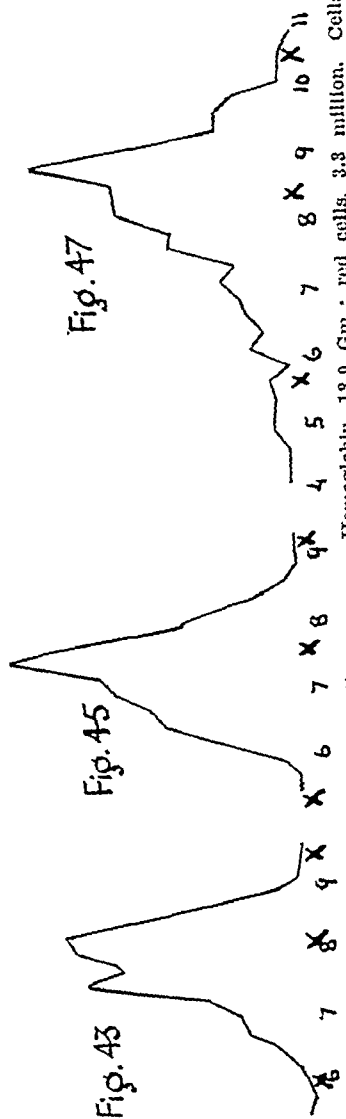


Fig. 41.—Obscure liver disease in native woman. Hemoglobin, 13.9 Gm.; red cells, 3.3 million. Cells enlarged, anisocytosis increased, good correspondence.

Fig. 42.—Pernicious anemia. Hemoglobin, 11.2 Gm.; red cells, 3.2 million. Cells very large, anisocytosis increased, very good correspondence.

Fig. 43.—Pernicious anemia. Hemoglobin, 13.1 Gm.; red cells, 3.9 million. Cells rather large, anisocytosis increased, very good correspondence.

Fig. 44.—Pernicious anemia. Hemoglobin, 7.0 Gm.; red cells, 2.5 million. Cells very large and extensive anisocytosis, rather more marked with diffraction method.

Fig. 45.—Anemia in native woman with syphilis. Mean diameter, normal, anisocytosis increased, rather more pronounced with diffraction method. Hemoglobin, 8.5 Gm.; red cells, 3.1 million.

Fig. 46.—Pernicious anemia. Hemoglobin, 10.9 Gm.; red cells, 3.2 million. Cells enlarged, anisocytosis increased, good correspondence.

Fig. 47.—Pernicious anemia. Hemoglobin, 8.0 Gm.; red cells, 2.7 million. Cells enlarged, but striking feature was very much increased anisocytosis, obvious by both methods.

Fig. 48.—Pernicious anemia. Hemoglobin, 10.2 Gm.; red cells, 3.5 million. Mean diameter normal, anisocytosis much



The advantages of this "duplication" technique and apparatus are compared with the various instruments suggested by other authors, which, although based on the same original idea, do not always do full justice to the possibilities of the diffraction method.

The main purpose of the paper is to show that with the diffraction method in the form finally developed, it is possible not only to determine the mean diameter, but also the degree and quality of anisocytosis present in a blood film. These determinations do not take more than a few minutes, which means a great saving in time as compared with the making of a Price-Jones curve. This diffraction method gives, in a fraction of the time, practically the same information as the laborious microscopic measurements of Price-Jones. It also takes into account a much larger number of cells. Its speed makes it readily applicable to any patient.

For differential diagnosis the importance of determining red cell diameters is admittedly great. The diffraction method in its proper form as described herein does this directly and very reliably. The indirect methods aiming at similar information by determining hemoglobin index or mean cell volume cannot be as reliable because it is necessary to divide two doubtful values through one another, and this may lead to gross distortion of actual conditions, as shown in this paper. Also, these indirect methods give mean values only, not degree and quality of anisocytosis.

The opportunity is taken of pointing out that blood films must be made with adequate skill and care to be of use in diffraction measurements. It seems that disappointments with the diffraction method in many instances are due to indifference in this respect.

In this paper are discussed the theoretical foundations of the diffraction method, and the technique of making accurate measurements is given in detail. A conversion table (Table III) is included which translates the diameters of the colored rings into microns, both for mean diameter and degree and quality of anisocytosis. This table is suitable for any apparatus working with a lens of 20 cm. focal distance.

A series of cases is described in which both microscopic measurements and complete diffraction measurements were performed, the identical blood films being used for both methods. It is seen that the results correspond quite well.

Finally, the usefulness of the diffraction method for following cases under treatment is illustrated.

#### PRACTICAL INSTRUCTIONS FOR BUILDING A DIFFRACTION APPARATUS

A suitable apparatus embodying the principles of Fig. 2 can easily be built, following the lines of the Pijper's Blood Cell Tester, of which an illustration is given in Todd and Sanford<sup>10</sup> and in other textbooks. One needs a wooden box, at the bottom of which is placed a small electric lamp, providing a narrow light source. A small circular diaphragm on top of the lamp produces a parallel beam of light, which reaches the horizontal stage of the apparatus, in which there are two small holes. (It would be a refinement to use a collector lens to provide a parallel beam.) In the Zeiss apparatus, contrary to the diagram, the beam strikes a simple lens, underneath the stage, before it reaches the blood films on the stage. This is the only lens which is essential. It is a simple lens of 20 cm. focal distance, mounted in a

5 diopters is good enough. The horizontal stage enables one to move the blood films until the best spot is found. The vertical partition which separates the two diffraction patterns should be made of a thin piece of metal. The receiving screen forming the top of the apparatus should be of fine grain ground glass; various kinds should be tried to get best results.

I wish to thank two of my technical assistants, Mrs. J. van Sighem and Miss J. Todd, for great care and patience exercised in the making of Price-Jones curves.

#### REFERENCES

1. Allen, A., and Ponder, E.: The Measurement of the Diameter of Erythrocytes by the Diffraction Method, *J. Physiol.* 66: 37, 1928.
2. Bergansius, F. L.: Die Messung von roten Blutkörperchen mittels der dadurch erzeugten Beugungserscheinungen, *Pflügers Arch.* 192: S. 118, 1921.
3. Bernstein, J.: Vergleich und praktischer Wert der Methoden zur Bestimmung des Erythrocytendurchmessers, *Folia haemat.* 52: 456, 1934.
4. Bock, H. E.: Ueber ein neues, einfaches Erythrocytenmessgerät für praktisch-klinische Zwecke (Erythrocytometer), *Klin. Wchnschr.* 12: 1141, 1933.
5. Christophers, S. R., and Craighead, A. C.: The Diffraction (Halometric) Method of Determining the Average Diameter of Red Blood Corpuscles, *Indian J. M. Research* 19: 963, 1932.
6. Edwards, W.: The Diagnosis of Anaemias by Diffraction, *Brit. M. J.*, Oct. 26, p. 785, 1929.
7. Emmons, W. R.: Clinical Eriometer, *Quart. J. Med.* 21: 83, 1927.
8. Eve, F. C.: The Early Diagnosis of Pernicious Anaemia by the Halometer, *Brit. M. J.* 2: 48, 1929.
9. Falisi, J. V.: Micro-diffractometer, *Am. J. M. Sc.* 186: 94, 1933.
10. Fullerton, H. W., Lyall, A., and Davidson, L. S. P.: The Diagnosis of Anaemia With Special Reference to Cell Volume and Blood-Iron Estimation, *Quart. J. Med.* 26: 561, 1933.
11. Haden, R. L.: A Comparison of Methods for Determining the Size of the Erythrocyte, *J. LAB. & CLIN. MED.* 10: 976, 1931.
12. Haden, R. L.: Diffraction Methods for Measuring the Diameter of the Red Blood Cell, *J. LAB. & CLIN. MED.* 23: 508, 1938.
13. Haden, R. L.: A New Instrument for the Diffraction Metric Measurement of the Diameter of Red Blood Cells, *J. LAB. & CLIN. MED.* 25: 399, 1940.
14. Hogben, H.: Apparatus for Measuring Mean Cell-Diameters in Blood Film, *Nature* 155: 576, 1945.
15. Hurst, A. F.: Addison's (Pernicious) Anaemia and Subacute Combined Degeneration of the Spinal Cord, *Brit. M. J.*
16. Knoche, E.: Untersuchungen am , , , nach Dr. A. Pijper, *Folia haemat.* 58: 212, 1937.
17. Menzies, W. F.: Remarks in Discussion on Mental Disorder and Pernicious Anaemia, *Brit. M. J.*, June 6, p. 980, 1931.
18. Millar, W. G.: The Diffraction Method of Measuring the Diameters of Erythrocytes, *Proc. Roy. Soc., London*, SB. 99: 264, 1926.
19. Pijper, A.: Diffraction Phenomena in Cultures of Microorganisms, *Med. J. S. Africa* 14: 211, 1918.
20. Pijper, A.: The Diffraction Micrometer, *South African M. Record* Sept. 27, 1919.
21. Pijper, A.: Diffraction Phenomena in Films of Blood Cells, *Med. J. S. Africa* 14: 472, 1919.
22. Pijper, A.: Diffraction-Phenomena in Films of Blood Cells and in Surface-Cultures of Micro-Organisms, *S. A. J. Sci.* 16: Oct.-Dec., 1919.
23. Pijper, A.: The Diagnosis of Addison's (Pernicious) Anemia by a New Optical Method, *Lancet* 2: 367, 1924.
24. Pijper, A.: Diffraction in Biological Structures. I, *South African M. Record*, June 9, 1923.
25. Pijper, A.: Diffraction in Biological Structures. II. Physical Conditions on the Surface of Bacteriological Agar Gels, *South African M. Record* 23: 222, 1925.
26. Pijper, A.: Diffraction in Biological Structures. III. The Relative Diameters of Members of the Coli-typhoid Group, *South African M. Record* 23: 453, 1925.
27. Pijper, A.: Diffraction in Biological Structures. IV. Blood Smears as Diffraction Gratings (With Special Reference to Addison's Anaemia), *South African M. Record* 23: 453, 1925.
28. Pijper, A.: Dimensional Differentiation of Certain Microbes, *J. M. A. S. Africa* 1: 203, 1927.
29. Pijper, A.: Liver Diet and the Red Cell Diameter in Addisonian (Pernicious) Anaemia, *J. M. A. S. Africa* 2: 483, 1928.



30. Pijper, A.: Eine neue Methode (Diffraktionsverfahren) für Durchmesserbestimmungen von roten Blutzellen, *Folia haemat.* 38: 320, 1929.
31. Pijper, A.: An Improved Diffraction Method for Diagnosing and Following the Course of Pernicious and Other Anaemias, *Brit. M. J.* 1: 635, 1929.
32. Pijper, A.: Red Cell Measurements: a Comparison of the Diffraction Method and the Microscopic Method, *J. Path. & Bact.* 34: 771, 1931.
33. Pijper, A.: The Zeiss "Pijper Blood-Cell Tester," *South African M. J.* 9: 205, 1935.
34. Pijper, A.: On Measuring Red Blood-Cells, With Special Reference to New Diffraction Apparatus, *Lancet* 1: 1152, 1935.
35. Ponder, E.: The Erythrocyte and the Action of Simple Haemolysins, Edinburgh, 1924, Oliver & Boyd.
36. Ponder, E.: The Mammalian Red Cell and the Properties of Haemolytic Systems, Berlin, 1934, Borntraeger.
37. Price-Jones, C.: The Diurnal Variation in the Sizes of Red Blood Cells, *J. Path. & Bact.* 23: 371, 1920.
38. Price-Jones, C.: The Sizes of the Red Blood Cells in Emphysema, *J. Path. & Bact.* 24: 326, 1921.
39. Price-Jones, C.: The Diameters of Red Cells in Pernicious Anaemia and in Anaemia Following Haemorrhage, *J. Path. & Bact.* 25: 487, 1922.
40. Price-Jones, C.: Red Cell Diameters in 100 Healthy Persons and in Pernicious Anaemia; the Effect of Liver Treatment, *J. Path. & Bact.* 32: 479, 1929.
41. Price-Jones, C., Vaughan, J. M., and Goddard, H. M.: Haematological Standards of Healthy Persons, *J. Path. & Bact.* 40: 503, 1935.
42. Pryce, D. M.: A Simplification of the "Halo" Method of Measuring the Diameter of Red Blood Corpuseles, *Lancet* 2: 275, 1929.
43. Renshaw, A., Twyman, F., and Follett, D. H.: Remarks in Discussion on Clinical Pathology, *Brit. M. J.*, Feb. 11, p. 229, 1933.
44. Schalm, L.: Le diamètre Moyen des globules rouges comme élément de diagnostic dans la différenciation des icteres, *Presse méd.* 48: 312, 1940.
45. Smith, K. E.: The Measurement of Red Blood Cell Diameter by the Diffractometer, *J. LAB. & CLIN. MED.* 26: 696, 1941.
46. Todd, J. C., and Sanford, A. H.: Clinical Diagnosis by Laboratory Methods, ed. 10, Philadelphia, 1944, W. B. Saunders Company.
47. Warburg, E. J., and Jørgensen, S.: Psychoses and Neurastheniae Associated With Achylia Gastrica and Megalocytosis, and the Relation Between This Syndrome and Pernicious Anaemia. I. Psychoses, *Acta med. Scandinav.* 69: 537, 1928.
48. Warburg, E. J., and Jørgensen, S.: Psychoses and Neurastheniae Associated With Achylia Gastrica and Megalocytosis, and the Relation Between This Syndrome and Pernicious Anaemia. II. Neurastheniae. Remarks on the Diagnostic Value of Color and Volume Indices, *Acta med. Scandinav.* 70: 195, 1929.
49. Wintrobe, M. M.: Clinical Hematology, London, 1942, Henry Kimpton.
50. Young, T.: An Introduction to Medical Literature, London, 1813, p. 545.

# THE ROLE OF PHAGOCYTOSIS IN RESISTANCE, AS RELATED TO AGE OF GRANULOCYTES FOLLOWING PRIMARY AND REINFECTION STUDIES WITH HEMOLYTIC STREPTOCOCCI IN MACACUS RIESUS

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IN THE studies previously reported from this laboratory<sup>1-3</sup> which attempted to analyze the relative roles of cellular and humoral immune factors in monkeys subjected to respiratory infections with the hemolytic streptococcus, Group C, not only the quantitative response of the neutrophilic granulocytes, but also the phagocytic activity (opsonocytophagic index of Huddleson<sup>4</sup>) in terms of relative cell maturity were investigated. To an initial intranasal infection with the streptococcus, there was invariably an immediate response marked by an effective granulocytic leucocytosis. A later reinfection with the same organism quite as regularly *failed* to elicit any appreciable disturbance in the quantitative equilibrium of the circulating white blood cells. Yet there was no apparent change in the effective resistance of the animals. It seemed desirable, therefore, to analyze the opsonic index under these two conditions, and at the same time to correlate the Arneth-Schilling index of maturity of the granulocytes to determine the optimum age for maximum phagocytic function under the conditions of this experimental study.

Previous studies by various workers concerning the qualitative and quantitative responses of the neutrophilic leucocytes to infection are not in agreement. Much of the discrepancy may be attributed to differences in technique and in the materials employed. Hamilton-Black<sup>5</sup> in 1913 observed the polymorphonuclear leucocytes during the tuberculin treatment of tuberculosis. After tuberculin he noted an increase in the number of mature cells as well as a total increase in leucocytes. Of the number of tubercle bacilli ingested (method not described) he found that neutrophils with one nucleus engulfed 10 per cent; with two nuclei, 18 per cent; with three nuclei, 22 per cent; and with four and five nuclei, 25 per cent, respectively. Arneth<sup>6</sup> in 1920, in examining purulent exudates but no peripheral blood preparations, reported that only mature leucocytes (Class 2 or 3) were concerned in phagocytosis, that the very young and the senile cells did not participate. Ponder and Flinn<sup>7</sup> in 1926 found no difference in the phagocytic properties of cells using *Mycobacterium tuberculosis*, *Escherichia coli*, *Staphylococcus aureus*, and *Neisseria catarrhalis*. Jacobsthal<sup>8</sup> in 1921 used citrated preparations from acute myeloblastic leucemia, myeloid leucemia, and Hodgkin's disease with multiple skin infiltrations and high eosinophilia. After

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finding staphylococci and the anthrax bacillus unsatisfactory, he used cinnabar and *Micrococcus tetragenus*. He concluded that:

1. Neutrophilic myeloblasts and myelocytes have the ability, in vitro, to phagocytize cinnabar and bacteria.

2. Eosinophiles are phagocytic.

3. Observations on the blood of two cases of leucemia showed that adult granulocytes but not myelocytes were able to phagocytize bacteria and foreign erythrocytes.

4. Eosinophiles in cases of Hodgkin's disease readily phagocytized staphylococci, micrococci, and tubercle bacilli.

Jung<sup>11</sup> in 1932 noted that as the number of cells increases, the percentage of phagocytes diminishes.

#### EXPERIMENTAL

*Materials and Methods.*—Daily determinations based on a modification of Huddleson's method<sup>6</sup> were made in selected monkeys in order to study any variation in the phagocytic properties of the circulating granulocytes of the infected animals. Young cultures of *Streptococcus hemolyticus*, Group C, which were culturally characteristic and not resistant to phagocytosis, were employed. For each opsonic determination, cultures grown on North's chocolate gelatin agar slants for eighteen to twenty-four hours at 37° C. were used. By means of a sterile wire loop the growth was gently removed from the surface and emulsified in sterile 2 per cent sodium citrate in physiologic saline solution which served both as a diluent and anticoagulant. The suspension was then adjusted by means of a nephelometer to a standard turbidity approximately equivalent to 30 billion bacteria per milliliter. After allowing a few minutes for any clumps or particles to settle, 0.1 ml. of the suspension was introduced into sterile agglutination tubes to which an equal amount of blood freshly obtained from the marginal ear vein was added. After being well shaken, the tubes were placed in a water bath adjusted to 37° C. The tubes remained in the bath approximately one hour when they were shaken again and then incubated an additional hour at 37° C. Duplicate thick smears of the incubated blood-bacterial suspension were made on clean glass microscopic slides and dried rapidly by means of an electric fan. The slides were then placed in a 5 per cent formalin and 1 per cent acetic acid solution for four minutes in order to dissolve the red corpuscles and to fix the leucocytes, following which the slides were dried with bibulous paper and the smears stained for thirty seconds with Bordet-Gengou's toluidine blue. In determining the degree of phagocytosis, the bacteria in twenty-five polymorphonuclear cells were counted and the phagocytic power measured by the average number of bacteria engulfed by each phagocyte in contrast with the number phagocytized in the presence of blood taken previous to inoculation. So far as possible, the individual cocci were counted. Daily blood counts were made on each animal over a period of two to three weeks. The age of each granulocyte in terms of lobation of the nucleus was recorded, and the engulfed bacteria counted.

*Experiment I.*—As noted in earlier studies<sup>1-5</sup> primary intranasal inoculation with the Group C streptococcus was followed by a sharp polymorphonuclear leucocytosis but the phagocytic index was not appreciably altered. Reinoculation, however, was followed by a sharp increase in individual phagocytosis but no leucocytosis occurred. Thus, in order to evaluate quantitatively the number of bacteria phagocytized daily, the total number of bacteria engulfed was determined by multiplying the opsonic index by the total number of neutrophils. The results obtained in the two monkeys to be discussed are representative of those obtained in a group of twenty-three animals studied.

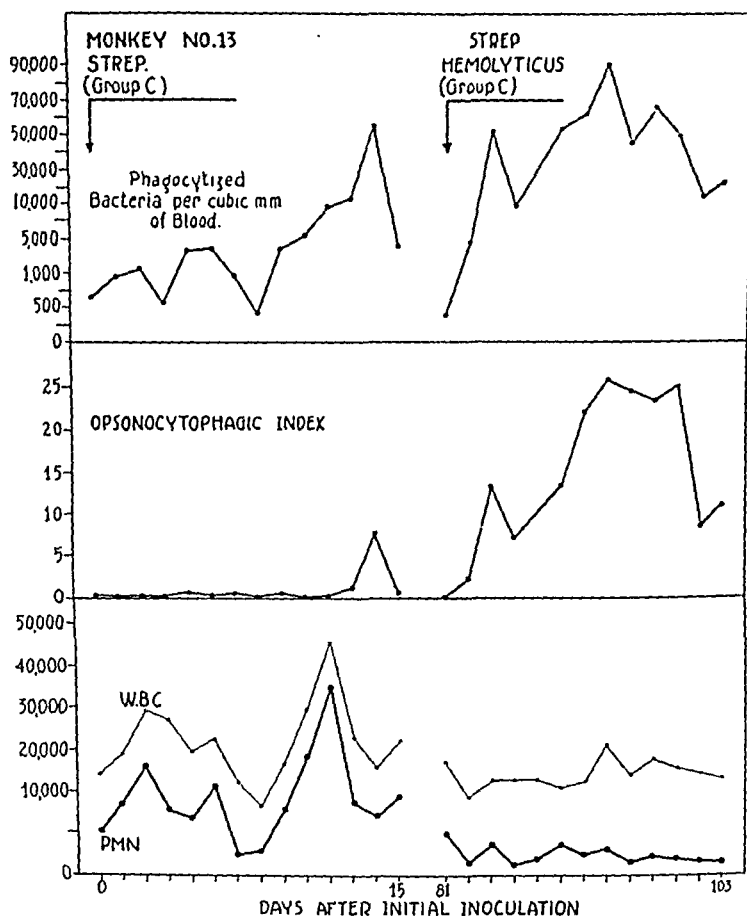


FIG. 1.

As noted in Fig. 1, Monkey 13 showed a marked polymorphonuclear leucocytosis after primary inoculation with relatively little increase in the opsonic index. However, there was a definite increase in the total number of bacteria engulfed from a preinoculation level of 672 bacteria per cubic millimeter of blood to a maximum of 55,600 bacteria on the twelfth postinoculation day, with intermediate levels varying from 456 to 13,338 bacteria per cubic millimeter. At

the time of maximum phagocytosis, the opsonic index was 8 as compared with 0.2 at the time of inoculation, while the neutrophils totaled 7,000 as compared with 4,000. On the tenth postinoculation day the total number of bacteria engulfed was 9,660 per cubic millimeter despite a low opsonic index of 0.3 due to the neutrophilic increase (34,500 polymorphonuclears); on the eleventh day there were 13,338 bacteria phagocytized per cubic millimeter when the opsonic index was only 1.5, but the neutrophils totaled 8,775.

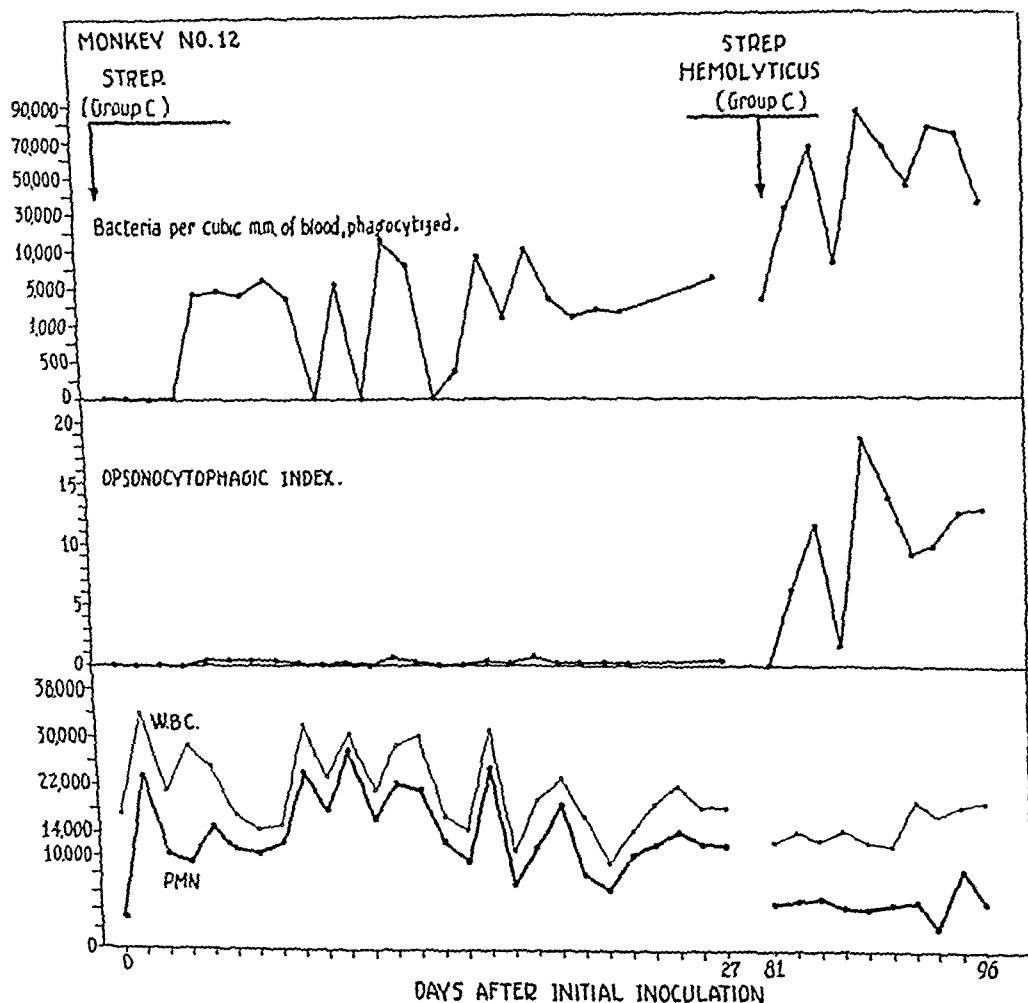


Fig. 2.

Following reinoculation, three months after primary infection, there was a consistently higher number of bacteria engulfed due primarily to the increase in individual cell phagocytosis without leucocytosis, with the highest number of bacteria engulfed being 87,000 on the seventh day after reinoculation with the streptococcus, at which time the opsonic index was 28 while total neutrophils were only 3,310. Twenty-four hours after reinoculation with the streptococcus

the opsonic index was 2.5 and the average number of bacteria phagocytized per cubic millimeter was 50,274; then it fell to 9,855 after forty-eight hours but increased on the third day to 50,557. For the next week the average number of bacteria phagocytized per cubic millimeter varied between 45,000 and 87,000, with opsonic indices ranging between 13.5 and 25.

Thus, although it was apparent that there was increased phagocytosis per individual cell following reinoculation as compared with primary inoculation with the streptococcus, due to the greater leucocytosis following primary inoculation there was actually less difference in the degree of total phagocytosis than would be expected from evaluation of the opsonic index alone.

Similarly, as noted in Fig. 2, there was a low opsonic index with a compensatory leucocytosis following primary instillation of the streptococcus, as compared with a lack of leucocytosis but with increased individual cell phagocytosis after reinoculation. Monkey 12 is also representative of the animals showing this response. The preinoculation opsonic index of 0 in this animal showed a slight increase to only 0.3 on the fifth day after primary inoculation with the streptococcus, with resultant indices for the next three weeks varying between 0 and 0.9. However, due to the leucocytosis there was an increase in total numbers of bacteria engulfed per cubic millimeter to as high as 17,700 on the twelfth day (opsonic index, 0.8; neutrophiles, 22,137), with the average number of bacteria engulfed per cubic millimeter being about 4,000. Before reinoculation with the streptococcus three months later, the opsonic index was 0.6; neutrophiles, 5,030; and the total number of bacteria engulfed per cubic millimeter, 3,018. Within twenty-four hours the opsonic index rose to 6.1, with 33,600 bacteria engulfed per cubic millimeter, although there was no increase in neutrophiles (5,510). For the next two weeks the opsonic index averaged between 6 and 18, except for one low value of 1.8 on the third day. The total number of bacteria engulfed per cubic millimeter rose to 83,600.

Here again there was a greater degree of phagocytosis per individual cell following reinoculation with the streptococcus than after primary inoculation. A greater total bacterial phagocytosis, however, followed this primary inoculation than might have been expected from the very low opsonic index.

*Experiment II.*—The monkeys used in this study were animals which had been infected by intranasal inoculation with the Group C hemolytic streptococcus three to six months earlier. After reinfection the granulocytes in these animals exhibited a marked increase in phagocytic activity which had not been apparent after primary infection, without the significant initial quantitative leucocytosis.

Fig. 3 presents graphically the relative degree of phagocytic activity by the various age groups of polymorphonuclear leucocytes. Monkey 8, for example, showed a progressive phagocytic efficiency beginning with the band forms (seventeen bacteria per cell), reaching a peak of twenty-two organisms per cell in the three-lobed neutrophiles, and then showing a gradually diminishing effectiveness in the four-, five-, and six-lobed cells which handled twelve, three, and six bacteria, respectively. Monkey 6 showed a similar picture with ten, thirteen,

and eleven bacteria engulfed by the one-, two-, and three-lobed cells, respectively, while the four-, five-, six-, and seven-lobed neutrophils each engulfed from four to seven bacteria.

The young cells in Monkey 13 likewise showed greatest phagocytic activity by the one-, two-, and three-lobed cells, while Monkey 17 showed a gradual progressive decrease in phagocytosis inversely proportional to the age of the leucocytes. The band cells in Monkeys 16, 29, and 30 had the highest phagocytic index with gradual diminution in phagocytosis as cell age increased.

In contrast, there apparently was no correlation between cell maturity and phagocytosis following primary infection. The small degree of phagocytosis observed here was apparently distributed at random among the various cells.

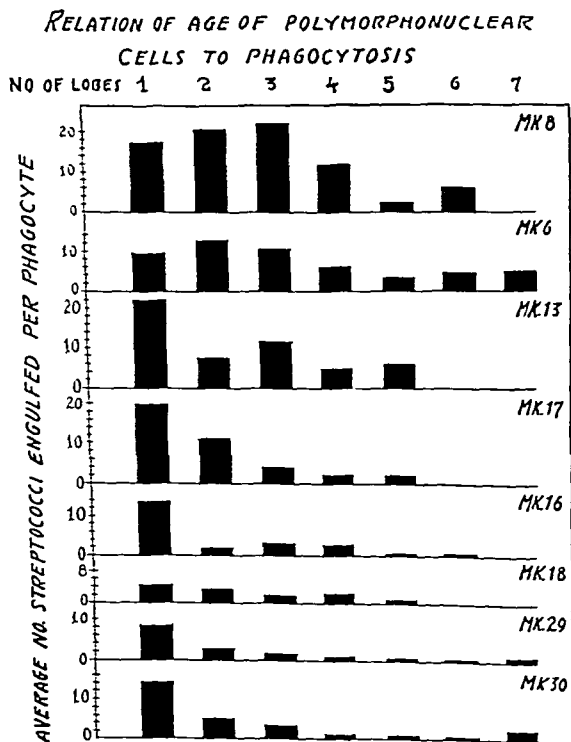


Fig. 3.

#### DISCUSSION AND SUMMARY

Earlier studies<sup>1</sup> from this laboratory have shown that monkeys receiving intranasal inoculations of *Streptococcus haemolyticus*, Group C, reacted with an immediate marked and clear-cut peripheral leucocytosis but without any elevation in the opsonic index as measured by *in vitro* tests. Reinoculation with the same agents three to six months later resulted in no appreciable leucocytosis, but there was a prompt and significant increase in phagocytosis by the individual granulocytes. If the *in vitro* determinations of the potential phagocytic capacities of the neutrophilic granulocytes for hemolytic streptococci multiplied

by their number per cubic millimeter in the circulating blood may be interpreted as a relative index of the cellular component of resistance to infection with these organisms, then the inverse ratio of these two phenomena, which occur in primary versus reinfection states, becomes not only interesting, but deeply significant as well. On the basis of the findings here presented, we agree with Jung,<sup>11</sup> that the lower the phagocytic index, the greater the number of granulocytes required to control any given infection. The absolute leucocytosis following close upon a primary infection with low opsonic index would seem to represent a compensatory response in which the resistance of the host or his cellular protection is measured by the sheer numbers of available phagocytes. When the relatively low opsonic indices are evaluated in relation to the total available polymorphonuclear leucocytes, the absolute number of bacteria phagocytized by the neutrophils will be greater than is at first evident from a cursory examination of the opsonic index alone. On the other hand, after reinoculation with the streptococci in these studies, there was a compensatory qualitative change in the phagocytic activity of the granulocytes without an absolute quantitative elevation in their number. In this group a qualitative cellular-humoral response apparently served as a compensatory protective mechanism by which fewer cells, each with greater individual phagocytic capacity, were able to control the infection effectively. Combining both the quantitative (leucocytic) and qualitative (phagocytic) responses, a greater total bacterial phagocytosis was noted following reinoculation with the streptococcus, but the difference with this cellular-humoral response was not as great as would be expected from evaluation of the opsonic index alone.

Wood<sup>12</sup> has recently called attention to the importance of phagocytosis of bacteria by neutrophilic granulocytes in bacterial pneumonia in the absence of demonstrable specific opsonins or antibodies. The motility of the cells in and through the fixed tissues brings direct contact with the invading organisms resulting in ready cytoplasmic inclusion. Our observations strongly suggest the acquisition of a highly specific humoral catalyst, which, upon reinoculation of the same pathogenic agent some months after recovery from the first invasion, greatly facilitates this phenomenon of spontaneous cellular phagocytosis, so that far fewer polymorphonuclears are needed to combat reinfection effectively the second time than the first.

The younger mature cells, according to the Arneth-Shilling index, were definitely more effective in their phagocytic activities in this study, with a decrease in phagocytic capacity as the individual cell age increased. These observations suggest that quantitative phagocytosis bears a direct relationship to the age and motility of the neutrophils. In the presence of opsonins, the more actively motile "left-shifted" cells have shown the greatest avidity in engulfing reinfecting pathogenic bacteria.

#### REFERENCES

1. Saslaw, S.: The Effect of Intranasal Inoculation of *Streptococcus Hemolyticus*, Group C, and Influenza Virus, Type A, in Monkeys on Adequate and Deficient Diets, Doctoral Dissertation, The Ohio State University, Columbus, 1942.



- 2 Schwab, J. L., Saslaw, S., Woolpert, O. C., Merino, C. and Doan, C. A.: Reactions of Monkeys to Experimental Respiratory Infections. I. Response to *Streptococcus Hemolyticus*, Group C. Proc. Soc. Exper. Biol. & Med. 48: 560, 1941.
3. Doan, C. A., Saslaw, S., Beard, M., Woolpert, O. C., Schwab, J. L. and Merino, C.: Reactions of Monkeys to Experimental Respiratory Infections. II. Response to Reinoculation With *Streptococcus Hemolyticus*, Group C, Proc. Soc. Exper. Biol. & Med. 48: 566, 1941.
- 4 Saslaw, S., Wilson, H. E., Doan, C. A., Woolpert, O. C., and Schwab, J. L.: Reactions of Monkeys to Experimentally Induced *Streptococcus Hemolyticus*, Group C, Infection. An Analysis of the Relative Roles of Humoral and Cellular Immunity Under Conditions of Optimal or Deficient Nutrition, J. Exper. Med. 84: 263, 1946.
- 5 Wilson, H. E., Saslaw, S., Doan, C. A., Woolpert, O. C., and Schwab, J. L.: Reactions of Monkeys to Experimental Mixed Influenza and *Streptococcus* Infections. An Analysis of the Relative Roles of Humoral and Cellular Immunity, With the Description of an Intercurrent Nephritic Syndrome, J. Exper. Med. 85: 199, 1947.
- 6 Huddleson, I. F., Hardy, A. V., Debono, J. L. and Giltner, Ward. Brucellosis in Man and Animals, ed 2, New York, 1943, Commonwealth Fund, Division of Publications, p 255
7. Hamilton Black, E.: The Qualitative and Quantitative Effect Observed on Polymorph Neutrophile Leucocytes in the Treatment of Tuberculosis by Tuberculin, Brit. M. J. 1: 113, 1913
8. Arneth, J. Die qualitative Blutlehre, Leipzig, Dr. Werner and Klinkhardt, 1920.
- 9 Ponder, E., and Flinn, Z. M. Studies on the Arneth Count. I The Relation Between Phagocytosis and Nuclear Configuration, Quart J Exper Physiol, Lond 16: 207, 1921
- 10 Jacobsthal, E. Ueber Phagocytoseversuche mit Myeloblasten, Myelocyten und Eosinophilen Leukocyten (mit Bemerkungen über den feineren Bau der Eosinophilen Leukocyten), Virchow's Arch. f. path. Anat. [etc.] Berl 234: 12, 1921.
- 11 Jung, R. W. Correlation Between Number of Leucocytes and Percentage of Phagocytosis, Proc Soc Exper Biol & Med 29: 981, 1932
- 12 Wood, W. B. IV. The Mechanism of Phagocytosis in the Absence of Antibody. J. Exper Med 84: 387, 1946

## OUTBREAK OF DIARRHEAL DISEASE ASSOCIATED WITH PARACOLON

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ORGANISMS of the incompletely circumscribed paracolon group are felt to be in a controversial position regarding their pathogenicity. A few outbreaks of diarrheal disease in human beings and in animals associated with paracolon flora in the intestinal tract have been recorded,<sup>1,2</sup> although usually it has been impossible to fulfill the postulates of Koch. Similarly, this report concerns an outbreak of diarrhea during which an organism of the paracolon group was isolated from the food consumed and from the stools of thirteen of the sixteen patients who required hospitalization.

In an Army officers' mess, fricasseed chicken was served to about 250 diners from 5:30 to 7:30 P.M. The chicken had been cooked in several large vessels and served directly from them. Approximately 100 servings were made from the one container of chicken which was found to contain paracolon bacteria when cultured later. There were eighty known cases of diarrhea; it could not be determined whether all these cases originated from the same batch of chicken. However, all patients had eaten chicken, and one had eaten nothing whatever except chicken.

About two hours after eating, the first patient became nauseated, had abdominal cramps, and soon began to vomit and pass watery stools. Other cases appeared between three and twelve hours after eating; the largest number of cases occurred four hours after eating the chicken. The five most severely affected patients developed mild shock which was relieved by simple measures. A total of eighty patients reported for treatment; how many were treated elsewhere could not be determined. Sixteen patients required hospitalization; these were the only ones available for culture during the acute illness. After twenty-four hours, all sixteen patients were symptom free except for weakness and had been discharged from the hospital. No sulfonamide therapy was given.

A rectal swab made from each hospital patient about nine o'clock in the morning after onset of the illness was planted on *Salmonella-Shigella* agar, on eosin-methylene blue agar, MacConkey's medium, and desoxycholate citrate agar and then placed in nutrient broth. Four hours later the nutrient broth was subcultured to eosin-methylene blue agar. No staphylococci were recovered from any of the cultures. In about eight hours, some *Salmonella-Shigella* agar plates showed heavy growth of lactose-nonfermenters. Almost all plates showed heavy growth of lactose-nonfermenters after twenty-four hours. Colonies were picked at twenty-four and forty-eight hours and, after replating, biochemical characteristics of the isolates were determined. Sugar fermentations in brom-cresol purple-nutrient broth were read daily for fourteen days. Indole forma-

tion was determined in tryptone water with Kovac's reagent after twenty-four hours of incubation. Citrate utilization was determined on Simmons' medium after twenty-four hours. Motility tests were done in 0.3 per cent agar and were read after five days of incubation at room temperature. Methyl red and Voges-Proskauer tests were done with the M.R.-V.P.\* medium. Gelatin liquefaction was read by refrigerating five-day cultures. The urease test of Rustigian and Stuart<sup>9</sup> was used.

The organism recovered from thirteen of the sixteen patients resembled paracolon 29911 of Stuart and co-workers<sup>10</sup> (Table I). It also bears a superficial resemblance to the genus *Proteus*, although it does not attack urea. It produces a bubble of gas in dextrose broth (which is not revealed after five days' incubation of a double sugar agar slant). Sucrose and mannitol are fermented in two to six days. Lactose, salicin, xylose, maltose, and rhamnose are not attacked in fourteen days. Methyl red and Voges-Proskauer reactions are negative, indole is produced, sodium citrate is utilized, urease is not formed, and the organisms are motile.

TABLE I

	DEXTROSE	LACTOSE	SUCROSE	SALICIN	MAITOSE	MANNITOL	INDOLE	V.P.	CITRATE	UREA	MOTILITY
29911-like strain	AB	-	A <sub>6</sub>	-	-	A <sub>4</sub>	+	-	+	-	++
29911 (Stuart)	AB	-	A <sub>6-20</sub>	-	-	-	+	-	+	- + w	++
<i>P. rettgeri</i>	AB	-	AG <sub>4</sub>	-	-	AG	+	-	+	+	++
<i>P. morganii</i>	AG	-	-	-	-	-	+	-	-	+ s	++
<i>P. vulgaris</i>	AG	-	AG	-AG	AG	-	+	-	-	+	++
<i>P. mirabilis</i>	AG	-	AG	-	-	-	-	±	+	+	++

AB, acid and a bubble of gas; A<sub>4</sub>, production of acid in six days of incubation; AG, acid and gas; -AG, some strains produce acid and gas, others do not; +, positive test or reaction; - + w, some strains negative, some weakly positive; + s, slow reaction; -, no reaction or negative test.

This organism differs from the one described by Stuart<sup>10</sup> as 29911 in that sucrose is fermented more rapidly (seven as against twenty days) and mannitol rather than maltose is attacked.

Two of the five strains isolated from the plates prepared from leftover chicken showed properties similar to this typical organism except that one produced a bubble of gas in sucrose broth in thirty days and the other was slow in developing motility.

Nineteen of the cultures from this outbreak (more than one isolate from some patients) have been found to be antigenically identical or very closely related. Further study of these cultures by Stuart has confirmed both biochemical and serologic connections with Type 29911 paracolon (Stuart). These strains may be classified as an atypical 29911 paracolon for the present.<sup>11</sup>

Rapid survey of the other strains isolated shows many related cultures and a large number not so apparently related. In addition to the paracolon described, *Proteus rettgeri* or *Proteus mirabilis* were isolated from twelve of the

\*Difco Laboratories, Inc., Detroit, Mich.

sixteen cases. *Proteus mirabilis*, together with the paracolon, was found in the chicken. Moreover, a large number of urease-negative organisms were recovered which differed in one or more respects from the 29911-like paracolon which was most prevalent.

## COMMENT

Careful application of standard methods to the study of an outbreak of common diarrheal disease revealed a paracolon as the predominating organism in the food implicated and in the stool cultures of the hospitalized patients. It appears unlikely that a recognized pathogen may have been overlooked. Whether the paracolon or the various *Proteus* species or the combination of them caused the outbreak cannot be definitely decided. A common pathogen occurring in the same circumstances would have been considered the etiologic agent. *Proteus rettgeri* and the 29911-like paracolon occurred together in several patients; it is noteworthy that the only important difference between the two is the ability of the former and the inability of the latter to attack urea. One is led to speculate as to their relationship, when they are associated in such a violent diarrhea.

## REFERENCES

1. Adams, J. W., and Atwood, R. T.: Bacillary Dysentery; a Bacteriologic and Clinical Analysis of 251 Cases Occurring in an Army Camp, War Med. 5: 14-20, 1944.
2. Barnes, L. A.: Pathogenic Enteric Bacilli; I. Paracolon, *Proteus*, and *Pseudomonas* Groups, U. S. Nav. M. Bull. 43: 707-716, 1944.
3. Cope, J. E., and Kilander, K.: Study of Atypical Enteric Organisms of the *Shigella* Group, Am. J. Pub. Health 32: 352-354, 1942.
4. Edwards, P. R., Cherry, W. B., and Bruner, D. W.: Further Studies on Coliform Bacteria Serologically Related to Genus *Salmonella*, J. Infect. Dis. 73: 229-238, 1943.
5. Fothergill, L. D.: Unusual Types of Non-Lactose-Fermenting, Gram-Negative Bacilli From Acute Diarrhea in Infants, J. Infect. Dis. 45: 393, 1929.
6. Peluffo, G. A., Edwards, P. R., and Bruner, D. W.: A Group of Coliform Bacilli Serologically Related to the Genus *Salmonella*, J. Infect. Dis. 70: 185-192, 1942.
7. Rhodes, A. J.: A Small Outbreak of Diarrhoea Associated With the Paracolon Bacillus, J. Hyg. 42: 99-102, 1942.
8. Stuart, C. A., and Rustigian, R.: Further Studies on One Type Paracolon Organism, Am. J. Pub. Health 33: 1323-1325, 1943.
9. Rustigian, R., and Stuart, C. A.: Decomposition of Urea by *Proteus*, Proc. Soc. Exper. Biol. & Med. 47: 108-112, 1941.
10. Stuart, C. A., Wheeler, K. M., Rustigian, R., and Zimmerman, A.: Biochemical and Antigenic Relationships of Paracolon Bacteria, J. Bact. 45: 101-119, 1943.
11. Stuart, C. A.: Personal communication.

# THE EFFECT OF THE RATE OF ADMINISTRATION OF AMINO ACID PREPARATIONS AND BLOOD AMINO ACID NITROGEN LEVEL ON THE PRODUCTION OF NAUSEA AND VOMITING

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THE intravenous administration of protein hydrolysates in the management of various protein deficiency states is now widely accepted. During the infusion untoward reactions occasionally occur which make it necessary to interrupt this form of supplementary protein therapy. These reactions include either one or any combination of the following signs or symptoms: Anorexia, nausea, vomiting, flushing, formication, headache, and dizziness.

It has been reported previously that under certain conditions the intravenous administration of casein digests produced a depressing influence on voluntary food intake.<sup>1</sup> In that study no direct relationship was found between the rate of administration and the degree of anorexia. Elimination of the other undesirable side effects could usually be accomplished by reducing the rate of the infusion. Clinically it is not desirable to subject a patient to prolonged infusion requiring three to four hours if it is possible to increase the rate of administrations without ill effects. For example, in order to give large amounts of these preparations by the intravenous route the patient will be required to remain inactive with one arm extended for long periods of time. The possibility of infection occurring at the site of the injection is greatly enhanced by the slow administration of these preparations.

In this hospital three different patients are known to have developed abscesses at the site of infusion, and five others showed clinical evidence of cellulitis following the slow rate of administration of a casein digest.

Hoffman, Kozoll, and Osgood<sup>2</sup> observed that nausea and vomiting occurred in patients who received a casein hydrolysate administered at rapid rates. These investigators are of the opinion that if the plasma amino acid nitrogen level exceeded 10 mg. per cent, nausea and vomiting usually occurred. During the course of previous studies<sup>1</sup> it was shown that a mixture of pure amino acids could be administered at exceedingly rapid rates without causing any undesirable effects.

The present investigations were undertaken to determine whether the relationship of the amino acid nitrogen content of the blood to nausea and vomiting, as proposed by Hoffman, Kozoll, and Osgood, existed with other amino acid preparations. In addition, the effect of the rate of administration on the production of these ill effects was studied.

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## METHODS

The patients used in this study were convalescent hospital subjects who had no known metabolic disorders. The intravenous injections were given as though they were a part of their regular therapy; thus the psychologic factors were minimized. The amino acid preparations were administered intravenously at rates varying from 5 to 100 ml. per minute, and if nausea or vomiting occurred a blood sample was immediately withdrawn from the opposite arm for amino acid nitrogen determination. In those patients in whom no symptoms occurred, a blood sample for amino acid content was taken just before the end of the infusion.

Five different amino acid preparations were used in this study. Preparation I\* was an enzymatic hydrolysate of casein which was administered as a 10 per cent solution. Preparations II† and IV† were acid digests of casein; the former was a 15 per cent solution and the latter a 6 per cent solution. Tryptophane was added to both Preparations II and IV, and, in addition, methionine was added to Preparation IV. Preparation III‡ consisted of a mixture of the ten "essential" amino acids plus the amino acid glycine. Approximately 50 per cent of the amino acids in this mixture were in the racemic form. Preparation V‡ was a vuj-n-type<sup>3</sup> mixture of amino acids prepared by the recombination and fortification of fractions of an acid hydrolysate of casein. It was administered as a 10 per cent solution. Of the total nitrogen in this mixture approximately 50 per cent was derived from essential amino acids.

Since the relationship between the amino acid nitrogen level of the blood and the presence of nausea and vomiting was one of the major interests of this study, no attempt was made to adjust the nitrogen concentrations of the preparations used. The volume of solution infused was the contents of either a 500 or a 1,000 ml. bottle. In the instance of Preparation II, however, due to the hypertonicity of the 15 per cent solution, 266 ml. of the protein hydrolysate were diluted to 500 ml. with sterile water and the resulting mixture administered.

The determination of the amino acid nitrogen content of the blood was made by the copper method of Albanese, and Irby,<sup>4</sup> all values being expressed as milligrams of amino acid nitrogen per 100 ml. of plasma.

## RESULTS

The influence of the rate of intravenous infusion on the occurrence of nausea and vomiting for all these preparations is summarized in Fig. 1. This factor was studied in 115 subjects. The casein digests (Preparations I, II, and IV) all had a high content of glutamic and aspartic acids, whereas the amino acid mixtures (preparations V and III) contained, respectively, little or none of these dicarboxylic acids. There appeared to be a slight increase in the incidence of nausea or vomiting when the rate of administration of preparation

\*Preparation I (Amigen, 10 per cent solution) was supplied by Mead Johnson and Company, Evansville, Ind.

†Preparations II (Parenamine) and IV (6 per cent Parenamine) were furnished by the Frederick Stearns & Company, Detroit, Mich.

‡Preparations III (Vuj) and V (Vuj-n-IX) were provided by Merck & Company, Inc., Rahway, N. J.

I was increased from 5 to 15 ml. per minute. In the case of Preparation II or IV, the increased rate of administration was usually accompanied by a marked increase in the incidence of nausea or vomiting. On the other hand, the administration of the amino acid mixtures, Preparation III or V, was accompanied by an exceedingly low incidence of ill effects. In this connection it is noteworthy that Preparation III has never made any patient ill, either in this or in earlier studies carried out in this hospital. The incidence of undesired side reactions was slightly higher when Preparation V was administered. It should be emphasized that the amino acid mixtures, Preparations III and V, were administered at increasing rates which began at the highest tolerated level for the protein hydrolysates. In many cases Preparation III was given at the rate of 500 ml. in eleven to thirteen minutes, whereas Preparation V was administered at the unusually rapid rate of 100 ml. per minute; thus, a 500 ml. bottle of this amino acid mixture was given in five minutes. In some of these cases flushing and formication occurred, but there was no nausea or vomiting.

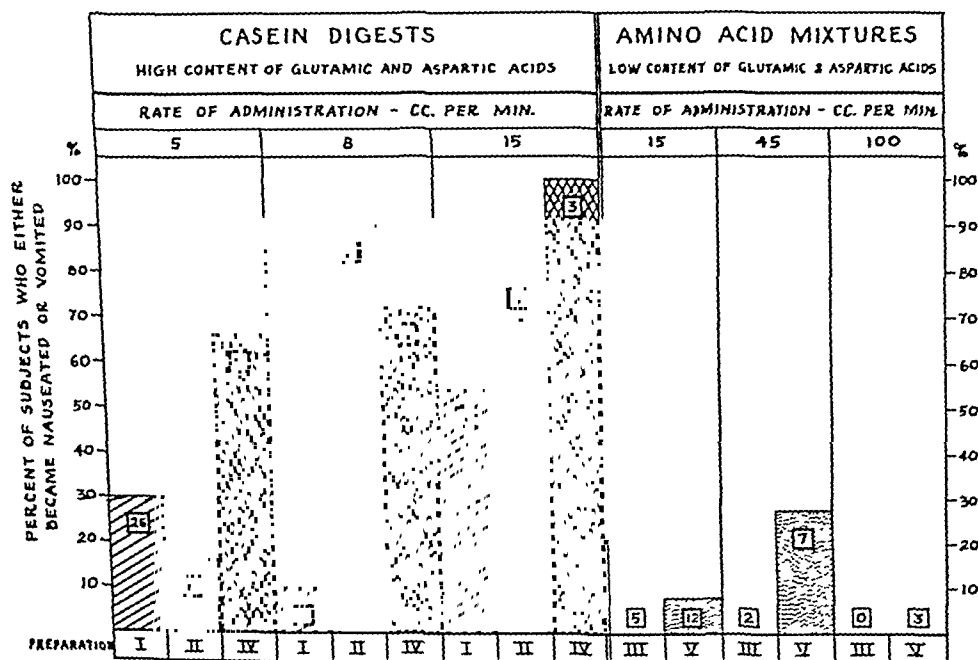


Fig. 1.—Influence of rate of administration on incidence of nausea or vomiting (115 subjects). The numbers in the squares at the top of each column indicate the number of subjects who received one of the five preparations shown along the bottom of the chart.

The relationship between the occurrence of vomiting and the level of the amino acid nitrogen in the blood plasma is summarized in Fig. 2. In this part of the study an attempt was made to vary the amino acid levels of the blood by varying the rate of the intravenous infusion. The administration of Preparation I caused no significant change in the incidence of nausea and vomiting until the amino acid nitrogen content of the plasma reached 13 mg. per 100 ml. or greater, at which levels 50 per cent of the patients became ill. With levels

below this, the frequency of nausea and vomiting ranged from 25 to 30 per cent of the patients studied. The highest amino acid nitrogen level in the plasma attained with this Preparation was 34 mg. per cent and in this subject only nausea occurred. When Preparation II was used, the incidence of nausea and vomiting increased progressively with an increase in the plasma amino acid nitrogen; if the amino acid nitrogen level of the plasma reached 9 mg. per cent or greater, all the subjects either became nauseated or vomited. With Preparation III there was no nausea or vomiting in any of the seven subjects reported in this study. In another series of fifteen patients who received forty-six infusions using this preparation, there also was no occurrence of any ill

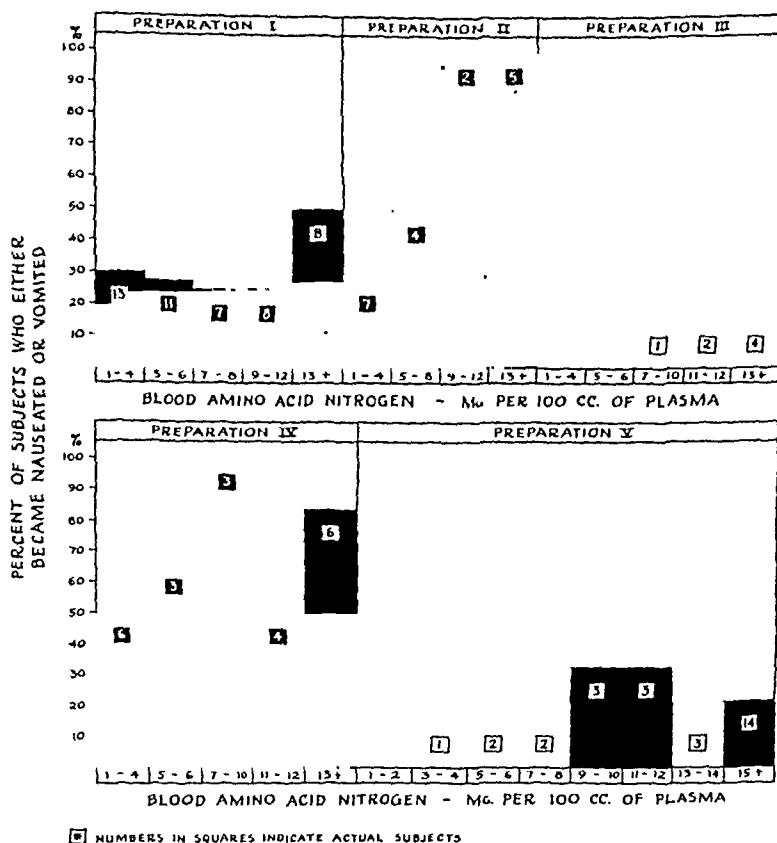


Fig. 2.—  
acid nitrogen  
bottom of the  
group of subjects

or vomiting in relation to preparations and blood amino  
The small numbers (from 1 to 15 or greater) along the  
range of blood amino acid nitrogen level in mg. per cent  
directly above.

effect. In a qualitative manner, Preparation IV reacted similarly to Preparation II, though the incidence of nausea and vomiting was not as great. Preparation V was administered at exceedingly rapid rates, and even at the highest plasma amino acid nitrogen level obtained, the incidence of nausea and vomiting was only 20 per cent. An amino acid nitrogen value of 44 mg. per cent was reached in one patient receiving Preparation V with no ill effects whatsoever.



## DISCUSSION

From the data presented in this investigation the incidence of nausea and vomiting appears to depend on the amino acid composition of the preparation used rather than on the average rate of administration or on the plasma amino acid nitrogen level. In all patients who received casein hydrolysates (Preparations I, II, and IV) the incidence of nausea or vomiting was greater than in those subjects who received the amino acid mixtures (Preparations III and V). In the case of Preparation II it was found that the presence of an amino acid nitrogen content in the plasma of about 10 mg. per cent or greater was usually associated with the occurrence of nausea or vomiting. This observation is in agreement with the work of Hoffman, Kozoll, and Osgood,<sup>2</sup> who used the same preparation.

The possibility that glutamic and aspartic acids are responsible for these toxic symptoms has been studied in both dogs and human subjects,<sup>5-8</sup> Preparation III was completely devoid of glutamic and aspartic acids and its use parenterally did not produce either nausea or vomiting. Preparations I, II, and IV are produced by hydrolyzing casein, a substance having a high content of these dicarboxylic acids, and therefore these digests are considered to have a large content of glutamic and aspartic acids. The glutamic acid content of Preparation V is 1.25 Gm. per liter or less.\* Thus the dicarboxylic acid content of this preparation is considerably lower than in mixtures resulting from the direct hydrolysis of casein. One explanation for the occurrence of nausea and vomiting resulting from the intravenously administered casein digests is that the blood dicarboxylic acid concentration becomes high enough to exceed a nausea threshold. The amino acid Preparations III and V, on the other hand, allow high blood amino acid nitrogen levels to be attained without nausea simply because little or no dicarboxylic acid is being added to the blood stream. Casein digests also differ from the amino acid mixtures by their content of nonamino acid substances which may be an additional factor contributing to the production of these ill effects. The possibility of a correlation between the plasma glutamic acid content and the presence of nausea and vomiting is being investigated.

Any evaluation of the toxic manifestations produced by the parenteral administration of amino acid preparations is difficult because the responses vary widely among apparently normal individuals.

## SUMMARY

The effect of five different amino acid preparations on the production of nausea and vomiting was investigated in human subjects. In all patients who received casein hydrolysates intravenously, the incidence of nausea or vomiting was greater than in those subjects who received the amino acid mixtures. Mixtures of amino acids can be administered intravenously at high rates without producing these effects. The casein hydrolysates, on the other hand, must be administered at considerably slower rates to avoid nausea and vomiting.

\*Personal communication from Dr. E. E. Howe, Merck & Company, Inc., Rahway, N. J.

With the mixtures of amino acids there appears to be no plasma amino acid nitrogen level at which nausea and vomiting uniformly occur. With casein digests, increased incidence of these ill effects is usually associated with high amino acid nitrogen values. The role of the dicarboxylic acid in the production of nausea and vomiting is discussed.

The production of nausea and vomiting by the intravenous administration of amino acid preparations depends on the preparation used and individual tolerance rather than on the degree of hyperaminoacidemia.

The authors wish to acknowledge the technical assistance of Dorothy L. DeZelia in this study.

#### REFERENCES

1. Smyth, C. J., Lasichak, A. G., and Levey, S.: The Effect of Oral and Intravenous Amino Acid Administration on the Voluntary Food Consumption in Normal Men, *J. Clin. Investigation*. In press.
2. Hoffman, W. S., Kozoll, D. D., and Osgood, B.: Blood Chemical Changes Following Intravenous Administration of a Casein Hydrolysate to Human Subjects, *Proc. Soc. Exper. Biol. & Med.* 51: 137, 1946.
3. Silver, R. H., Seeler, A. O., and Howe, E. E.: Urinary Excretion of Amino Nitrogen Following Intravenous Administration of Amino Acid Mixtures, *J. Biol. Chem.* 164: 639, 1946.
4. Albanese, A. A., and Irby, V.: Determination of Amino Nitrogen of Blood Filtrates by the Copper Method, *J. LAB. & CLIN. MED.* 30: 718, 1945.
5. Madden, S. C., Woods, R. R., Shull, F. W., Remington, J. H., and Whipple, G. H.: Tolerance to Amino Acid Mixtures and Casein Digests Given Intravenously; Glutamic Acid Responsible for the Reactions, *J. Exper. Med.* 81: 439, 1945.
6. Unna, K., and Howe, E. E.: Toxic Effects of Glutamic and Aspartic Acid, *Federation Proc.* 41: 138, 1945.
7. Smyth, C. J., Lasichak, A. G., and Levey, S.: Glutamic Acid as a Factor in Nausea and Vomiting During Intravenous Amino Acid Alimentation, *Proc. Central Soc. Clin. Research* 19: 56, 1946.
8. Smyth, C. J., Levey, S., Lasichak, A. G.: The Relationship of Glutamic and Aspartic Acids to the Production of Nausea and Vomiting in Man, *Am. J. M. Sc.* In press.

## STUDY OF A NEW SEDATIVE-HYPNOTIC DRUG (3,3-DIETHYL-2,4-DIOXOTETRAHYDOPYRIDINE)

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WHILE there is an abundance of sedatives and hypnotics available to physicians, none is completely satisfactory. In fact, even those which are most popular with the profession occasionally elicit untoward side reactions such as drowsiness, "hangover," excitation, and delirium, as well as allergic responses involving chiefly the skin. Such toxic effects are especially encountered with the barbiturates. Thus, there is a place for a mild hypnotic preparation which is distinguished by a minimum of side-reactions and which chemically does not belong to the barbiturates.

In the light of the foregoing it was decided to undertake the clinical appraisal of a sedative-hypnotic compound (3,3-diethyl-2,4-dioxotetrahydropyridine) which appeared to afford certain advantages over the barbiturates, as substantiated by findings of Koppányi, Herwick, Linegar, and Foster.\* These investigators explored the pharmacology of the drug extensively, and some of their observations are summarized here.

The minimal lethal dose of the aforementioned pyridine compound for rabbits was 500 to 650 mg. per kilogram orally and 300 to 350 mg. per kilogram intravenously. In aqueous solution the drug was readily absorbed following oral, intramuscular, subcutaneous, and intraperitoneal administration. The smallest oral dose required in the rabbit for motor paralysis and sensory depression was 250 mg. per kilogram. Sleep was induced within ten minutes when 150 mg. per kilogram were given intramuscularly or intraperitoneally to rabbits, but by the subcutaneous route this dose produced only drowsiness. Motor incoordination, the first sign of effect of the drug, occurred usually within one or two minutes following intramuscular or intraperitoneal injection. In two rabbits receiving daily 250 mg. per kilogram parenterally for seven days, there was no evidence of accumulation. The animals eliminated at least five-sixths of the dose within twenty-four hours. With regard to the duration of action, it was shown that rabbits eliminated, during an average period of five and one-third hours, about 70 per cent of the intravenous fatal dose; that is, about 13 per cent per hour. The average sleeping time from approximately 60 per cent of the minimal lethal dose was found to be as follows: eight hours (rabbits, orally), three and one-half hours (rabbits, intravenously), and five hours (dogs, intravenously). There was great individual variation in recovery time but no evidence of acquired tolerance.

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\*Koppányi, T., Herwick, R. P., Linegar, C. R., and Foster, R. H. K.: Studies on the Pharmacology of 3,3-Diethyl-2,4-Dioxotetrahydropyridine, and 3,3-Diethyl-2,4-Dioxopiperidine, *Arch. internat. de pharmacodyn. et de thérap.* 64: 123-152, 1910.

TABLE I

RABBITS ON INTRAVENOUS ADMINISTRATION	3,3-DIETHYL-2,4-DIOXOTETRAHYDRO-PYRIDINE	BARBITAL
Narcotic dose for 50% of animals (ND50) in mg./kg.		
Lethal dose for 50% of animals (LD50) in mg./kg.	86	140
	350	415

TABLE II

MICE ON SUBCUTANEOUS ADMINISTRATION	3,3-DIETHYL-2,4-DIOXOTETRAHYDRO-PYRIDINE	PHENOBARBITAL
Narcotic dose for 50% of animals (ND50) in mg./kg.		
Lethal dose for 50% of animals (LD50) in mg./kg.	200	222
	1,062	473

Tables I and II derived from data of the report by Koppányi and co-workers give some of the comparative results on the experimental pyridine compound, barbital, and phenobarbital.

From these figures it appears that intravenously in rabbits, the narcotic index (LD50/ND50\*) of 3,3-diethyl-2,4-dioxotetrahydropyridine is 4.07 and that of barbital is 2.96. Similarly, on subcutaneous administration in mice the narcotic index of the experimental drug is 5.31, but that of phenobarbital is 2.13. It can be concluded that the margin of safety of the pyridine compound ranks decidedly higher than that of either barbital or phenobarbital.

Systemic reactions to 3,3-diethyl-2,4-dioxotetrahydropyridine were similar to those obtained with barbiturates; namely, a fall in blood pressure, depression of respiration and body temperature, and an increase in pulse rate. In a number of other animal experiments it was shown that the pyridine compound behaved typically of a true hypnotic by preventing convulsions from strychnine and picrotoxin. In dogs there was no appreciable effect on the blood sugar except after large amounts of the drug. Rabbits receiving twenty-five to eighty-six daily oral doses of from 15 to 400 mg. per kilogram of the pyridine derivative showed no deviations from normal in general appearance, food consumption, and reaction to other drugs. Likewise, there was no evidence of the development of tolerance, sensitization, or addiction in these animals, and changes in the blood picture were within normal limits.

With this pharmacologic evidence of effectiveness and low toxicity, clinical trials with 3,3-diethyl-2,4-dioxotetrahydropyridine† were instituted.

Obviously, the evaluation of therapeutic results with a sedative-hypnotic is open to a wide range of interpretation since the subjective response of patients to such medication depends upon many factors, some of which are beyond the

\*LD50 is defined as the lethal dose for 50 per cent of the animals and ND50 as the narcotic dose which prevents a righting response in 50 per cent of the animals after the tails are pinched.

†3,3-diethyl-2,4-dioxotetrahydropyridine was furnished by Hoffmann-La Roche, Inc., Nutley, N. J., under the designation NU-903, through the courtesy of Dr. Leo A. Pirk.

control of the experimenter. Nevertheless, an attempt was made to evaluate the effects as objectively as possible. Thus relief from complaints was not suggested or promised to the patients, and the reports of responses to the drug were obtained as casually as possible. In fact, the patients were encouraged to volunteer their impressions. Furthermore, a relatively large number of subjects were tested so that many of the potential psychic factors which might influence the drug's appraisal could be neutralized.

NU-903 was administered to two groups of patients: one consisted of subjects suffering from insomnia, and the other of persons requiring daytime sedation because of nervous tension. Among the latter were patients with a variety of psychosomatic disorders.

In the first group there were ninety-six patients who had all complained that they slept poorly or fitfully. Of these, sixty-six received one tablet of NU-903 (0.2 Gm.) at bedtime; forty-two, including twelve who had failed to respond to the aforementioned dosage, were given two tablets (0.4 Gm.), all for at least seven consecutive nights. An uninterrupted period of trial makes changes more perceptible, and thus these patients were in a better position to appraise the effect of the drug on their sleep than if they had taken it only sporadically. The higher dosage was administered in those instances in which clinical appraisal indicated a more severe degree of insomnia or a more agitated mind.

The effects of this treatment are shown in Table III. The degree of the positive responses is indicated by the number of plus marks: three signs denote excellent; two signs, satisfactory; and one sign, fair results. Negative responses are designated by 0.

TABLE III. TREATMENT OF INSOMNIA WITH NU-903

DOSAGE	NUMBER OF PATIENTS	TOTAL NUMBER OF TREATMENT	AVERAGE ONSET OF EFFECT	AVERAGE DURATION OF SLEEP	RESULTS
0.2 Gm.	66	482	30 min.	6 hr.	10 + + +
					21 + +
					24 +
					11 0
0.4 Gm.	42	478	30 min.	7 hr.	10 + + +
					22 + +
					6 +
					4 0

It is apparent that 0.2 Gm. of NU-903 was not quite sufficient to induce satisfactory sleep in the majority of patients, although it did have a beneficial effect on many. It seems as though the desired therapeutic results were obtained with this dose only in persons who were sensitive to hypnotic drugs or whose insomnia was of a mild degree. Furthermore, it is evident that 0.4 Gm. of NU-903 produced excellent or satisfactory sleep in thirty-two of forty-two patients. (Among those reporting gratifying results on 0.4 Gm. were twelve patients in whom 0.2 Gm. had failed to induce satisfactory sleep.) Six patients claimed to have had only a fair degree of response with 0.4 grams. Finally, of the four subjects

who reported no results with this dose, three had previously experienced insufficient hypnotic effect from 0.1 Gm. of sodium pentobarbital, but they did have a good night's sleep from 0.2 Gm. of the latter somnifacient. From these results it may be concluded that 0.4 Gm. of NU-903 will produce a hypnotic effect comparable to that of 0.1 Gm. of sodium pentobarbital, the standard dose.

The onset of sleep, regardless of the dose, followed taking of the drug usually within thirty minutes, but never after more than sixty minutes. The duration of sleep averaged six hours following 0.2 Gm. of NU-903 and seven hours following 0.4 Gm., with those patients being taken into consideration who reported excellent or satisfactory results.

One subject receiving 0.2 Gm. and one receiving 0.4 Gm. complained of drowsiness upon awakening. Otherwise, there was a complete lack of untoward subjective reactions. Similarly, neither allergic phenomena nor other signs of idiosyncrasies were observed. This relative freedom from undesirable side effects offers a distinct advantage over the barbiturates, as evidenced by the fact that among the ninety-six patients treated for insomnia with NU-903 there were six persons who had experienced untoward reactions to barbiturates, such as excitability, excessive drowsiness, or skin reactions.

In the second group receiving NU-903 for daytime sedation, there were eighty-seven patients. About 60 per cent of these took amphetamine sulfate (30 mg. a day) for the purpose of weight reduction, and in these the experimental drug was given to overcome the nervous excitation induced by this stimulant.

Thirty-five patients received one-half tablet of NU-903 three times daily (total dose 0.3 Gm.), and fifty-two were given one tablet three times daily (total dose 0.6 Gm.). At the lower dosage level the average number of treatment days was fifteen, and at the higher dosage level it was twenty-one. However, eighteen patients received 0.2 Gm. three times daily for thirty days, and three for as long as ninety days.

The effects of this treatment are shown in Table IV. The degree of favorable responses was more difficult to ascertain than in the group receiving the drug as a soporific, and the therapeutic effects were therefore listed in a broader manner, by classifying them as satisfactory, fair, or none.

TABLE IV. TREATMENT OF NERVOUS TENSION WITH NU-903

DOSAGE	NUMBER OF PATIENTS	TOTAL NUMBER OF TREATMENT DAYS	RESULTS
0.1 Gm. t.i.d.	35	525	22 Satisfactory
			10 Fair
			3 None
0.2 Gm. t.i.d.	52	1,092	36 Satisfactory
			10 Fair
			6 None

As can be seen from Table IV, beneficial results were obtained in the majority of patients taking either 0.1 Gm. or 0.2 Gm. three times daily. The incidence of ineffectiveness of the drug as a sedative was about 10 per cent. It is not

easy to compare the responses to NU-903 with those to other sedatives because of the lack of objective criteria for such a comparison. Nevertheless, in eighteen patients who had received 0.2 Gm. of NU-903 three times daily, 30 mg. of phenobarbital, also given three times daily, were substituted for the experimental preparation. Six of these subjects claimed that they felt a greater degree of relaxation of nervous tension when they received NU-903. These observations give the tentative impression that the sedative effect of 200 mg. of NU-903 is somewhat superior to that of 30 mg. of phenobarbital. It may be inferred that on a weight basis, about four to five times as much of NU-903 as of phenobarbital has to be given in order to obtain the same degree of sedation. Untoward subjective reactions were conspicuous by their absence in all eighty-seven patients of this group. Likewise, there were no signs of idiosyncrasy. In this connection it is noteworthy that five of these eighty-seven subjects had previously reacted with a pruritic rash to barbiturate medication.

For ten of the patients receiving 0.1 Gm. of NU-903 three times daily for two weeks (included in Table IV), pre- and postmedication blood counts were done, including red, white, and differential counts, as well as hemoglobin determinations. There was no significant change in the blood picture of any of the subjects. In addition, a collateral study of a similar nature was carried out in nine persons not requiring sedative medication and, therefore, not included in the series that served for clinical evaluation of the experimental preparation. Each of these subjects received 0.1 Gm. of NU-903 three times daily for periods ranging from twenty-two to sixty-one days. The average number of treatment days was 38.2, and the total doses taken by the individual persons during the period of observation were as follows: 18.3, 12.6, 9.9, 12.9, 9.3, 6.6, 12.6, 13.8, and 7.2 grams. Hemograms, including differential counts and hemoglobin determinations, done at initiation and termination of medication revealed that there were no significant changes in the quality of the blood in any of the nine subjects.

#### SUMMARY AND CONCLUSIONS

A new sedative-hypnotic drug, 3,3-diethyl-2,4-dioxotetrahydropyridine, tentatively designated NU-903, was evaluated clinically in 183 patients. There were two groups of subjects: to one group, consisting of ninety-six patients, close to 1,000 doses of the drug were given to induce hypnosis, and to the other, consisting of eighty-seven patients, the preparation was administered as a daytime sedative for a total of over 1,600 treatment days. The results were as follows:

Four-tenths of a gram produced excellent or satisfactory sleep in thirty-two of forty-two patients. A dose of 0.2 Gm. proved less gratifying as a somnifacient, judged by results recorded for sixty-six patients. Of these, only thirty-two responded with excellent to satisfactory sleep, and the rest had poor or no results. However, in twelve in whom the lower dosage failed to produce gratifying results, satisfactory sleep was induced by 0.4 grams. In all subjects medication was given for at least seven consecutive nights.

For treatment of nervous tension, 0.1 Gm. of NU-903 three times daily was prescribed to thirty-five patients (average number of treatment days, fifteen).

and 0.2 Gm. three times daily to fifty-two individuals (average number of treatment days, twenty-one). Adequate sedation was obtained at both dosage levels in the majority of patients; there was no sedative effect in 10 per cent of the cases.

One subject receiving 0.2 Gm. and one receiving 0.4 Gm. as a somnifacient complained of drowsiness upon awakening. Otherwise, that is, in 181 patients, there was complete absence of untoward subjective reactions. No allergic phenomena were observed in any of the 183 cases treated. In contrast, eleven of these subjects experienced undesired aftereffects following barbiturate medication.

Blood studies carried out in nineteen subjects receiving 0.1 Gm. of NU-903 three times daily over prolonged periods of time did not reveal any untoward effects of the drug on the quality of the blood.

It can be concluded that NU-903 is an effective hypnotic and sedative which is distinguished by excellent tolerability.



# LABORATORY METHODS

## THE ASSAY OF HEPARIN

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NUMEROUS methods for the standardization of heparin using citrated or oxalated whole blood or plasma have been described in the literature. Because of peculiarities in the development of clots *in vitro*, and the difficulty in distinguishing one from another, several means of determining an end point have been suggested. In Foster's<sup>1</sup> modification of the Reinert and Winterstein method, as many as eleven evaluations for various clot formations, ranging from a full clot to no clot, have been proposed. The assigned values are plotted logarithmically to determine the theoretic one-half clot. The method of Howell, as modified by Charles and Scott,<sup>2</sup> calls for the use of cat blood. Clot formations occurring in the standard heparin tube series are matched against those seen in the unknown series. Large differences in the clotting nature of cat blood renders difficulty in duplicating results. The method of Jacques and Charles<sup>3</sup> utilizes oxalated beef blood with the addition of thrombin. Impurities in heparin samples interfere with the clotting mechanism and usually have a tendency to exaggerate their anticoagulant activity. The Fischer and Schmitz<sup>4</sup> procedure using chicken blood does not permit the repetition of the assay under identical conditions. Kuizenga, Nelson, and Cartland<sup>5</sup> report that recalcified sheep plasma requires a smaller increment in heparin for a transition from coagulation to fluidity than either recalcified beef or horse plasma. They report that 2  $\mu\text{g}$  (0.2 Toronto unit) of standard heparin containing 100 Toronto units per milligram are necessary for this complete change. In the method herein described, only 1  $\mu\text{g}$  of such standard heparin is required to effect this change for recalcified beef or sheep plasma.

The evaluation of clots according to opacity, granulation, density, adherence to the walls of test tubes, traces, etc., is difficult. Values so assigned are subject to personal error of judgment and can lead to an erroneously plotted or matched end point. Experience has shown that two persons seldom evaluate or match clots identically. Also, slight modifications in the amounts of calcium, plasma or whole blood, saline, temperature, or time affect the development of clots to a noticeable degree. My experience is that a difference of as little as 0.05 mg. of calcium or 0.1  $\mu\text{g}$  of heparin between two tubes containing 1.0 ml. of diluted citrated plasma or whole blood can cause an appreciable difference in clot formation. Such slight errors can easily be made in an assay, and their combined effects may be additive in determining the development of a clot.

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My several years' experience with Foster's method, while working with him, has indicated possibilities for a simplified and improved heparin assay which are incorporated in the method herein described. The increment of heparin in each tube in a series is very small and constant, since only 1.0  $\mu$ g. of the standard heparin is necessary to pass from clot to fluidity. The time which elapses between the addition of the calcium to the plasma is reduced to a minimum so that all tubes are treated identically. The end point is the least amount of heparin necessary to prevent any clot formation; it is definite and reproducible. Fresh or frozen<sup>6</sup> citrated plasma can be used with equal accuracy.

#### PROCEDURE

*Plasma.*—Fresh beef blood is obtained in any desired quantity at the slaughterhouse. Flasks for holding the blood are prepared in the laboratory by placing therein 100 ml. of an 8 per cent sodium citrate solution and marking them at a 2-liter level. The blood is collected from the animals in paraffin-coated pitchers and immediately poured into the flasks to the 2-liter mark. The flasks are then stoppered and agitated gently. Upon reaching the laboratory, the blood is centrifuged at 2,000 r.p.m. for thirty minutes and the supernatant plasma is siphoned off and pooled. Each heparin sample to be assayed requires 12 ml. of plasma. Therefore, quantities slightly in excess of the amount required for an assay consisting of the standard heparin and any desired number of heparin samples are filled into Florence flasks. The flasks should be filled to approximately two-fifths of their capacity. They are then corked, frozen rapidly by rotation in a tilted position in an acetone dry ice bath, and stored in a refrigerator at  $-20^{\circ}$  C.

*Calcium Chloride Determination for Clotting.*—The optimum calcium chloride level for recalcifying the plasma in this procedure must be determined for each new lot of fresh or frozen citrated plasma. This level is the amount of calcium chloride necessary to permit the end-point tube (the tube in which any clot formation has just been prevented by the heparin) in the standard heparin series to be located near the middle of the series. This quantity of  $\text{CaCl}_2$  must be contained in a 0.2 ml. volume of physiologic saline which is used for each tube throughout the assay. The standard heparin should contain at least 100 Toronto units or 500 anticoagulant units<sup>7</sup> per milligram.

When blood is citrated as previously described, usually 0.95 mg. of calcium chloride is necessary for the recalcification. However, the amount may vary slightly and can be determined in the following manner: To six tubes containing 0.35 ml. of a 10  $\mu$ g per milliliter standard heparin solution is added, from the first to the last, 0.16, 0.18, 0.19, 0.20, 0.21, and 0.22 ml., respectively, of a 5 mg. per milliliter calcium chloride solution. Saline is added to bring the volume to 1.0 milliliter. Add 1.0 ml. of plasma and stopper with corks which have only the inserted face coated with paraffin (this prevents the corks from slipping out), invert two or three times, and place in a water bath at  $37^{\circ}$  C. for three hours. Then examine the tubes and select the one with the

most amount of calcium chloride having no clot formation. The amount of calcium chloride added to that tube is to be contained in the 0.2 ml. volume of physiologic saline mentioned. The concentration so determined can be used for all subsequent assays, using portions of the same plasma.

*Assay.*—The standard heparin powder and unknown finished powders are made up in concentrations of 10  $\mu\text{g}$  per milliliter. Impure heparin powders, generally low in potency, are made up in concentrations of 20 or 40  $\mu\text{g}$  per milliliter, depending upon their activities. One or two assays may be necessary to determine the concentration to use. Heparin vial solutions are diluted to a concentration of 1.0 Toronto unit per milliliter. Physiologic saline is used for all solutions. Acid-cleaned glassware is a prerequisite for an accurate assay. A series of twelve tubes (10 by 100 mm.) is used for the standard heparin and for each sample to be assayed. Microburettes are used for measuring the plasma and all solutions. To the first tube of the standard heparin series is added 0.23 ml. of the standard heparin solution; in each subsequent tube the amount of heparin solution added is increased by 0.02 ml. over the preceding tube so that the twelfth tube will contain 0.45 milliliter. Each unknown heparin solution is likewise measured into a series of twelve tubes. Saline is then added to all tubes to bring the volumes to 0.8 milliliter. A constant sequence is kept in adding the plasma and calcium chloride solution. To the first tube of each series add 1.0 ml. of plasma and 0.2 ml. of the calcium chloride solution; stopper with paraffin-coated corks, invert two or three times, and place in a water bath at 37° C. for three hours. Repeat this procedure for the second tube of each series and each subsequent tube until all the tubes have been so treated. By doing this, the influence of plasma changes is minimized. After three hours, the tubes are observed. That tube in each series in which any clot formation has just been prevented is the end-point tube for that series. The use of fluorescent light has been found most desirable for the detection of small clot formations. Very often a thin film of clot formation adheres to the rounded bottom of the tube; this is detected by inverting and uprighting the tubes rapidly once or twice in front of the lamp. The amount of heparin in the end-point tubes is used to calculate the potency of the unknown heparin represented by that series, in terms of the standard heparin.

*Example.*—The standard heparin potency is 110 Toronto units per milligram. To prevent clot formation, 3.1  $\mu\text{g}$  of the unknown heparin powder and 2.7  $\mu\text{g}$  of the standard heparin are required: the strength of the unknown heparin is 95.8 Toronto units per milligram.

$$\frac{110 \times 2.7}{3.1} = 95.8 \text{ Toronto units per milligram.}$$

If in this example, the unknown represented a heparin vial (10 mg. per cubic centimeter), diluted 1,000 times, the potency of 1.0 ml. of the original vial solution would be

$$\frac{110 \times 2.7}{3.1} \times 10 = 958 \text{ Toronto units per milliliter.}$$

## DISCUSSION

The small increment of heparin permits an end point to be approached with a greater degree of accuracy than has heretofore been possible in other heparin assays. The increment makes it possible to detect with ease slight errors in titration in following the procedure because the diminution of the clot to fluidity is remarkably uniform in the series. This transition range, under the specific conditions of the assay procedure presented in this paper, requires only 0.1 Toronto unit of activity (1  $\mu$ g of standard heparin). Fresh and frozen citrated beef and sheep plasma have been used with the same degree of accuracy, and the transition from clot to fluidity requires approximately the same heparin activity. It is, however, easier to determine traces of clot formation in beef plasma than in sheep plasma. Also, it is easier to obtain beef plasma, in large quantities, than sheep plasma, and the results obtained are more uniformly duplicated. Repeated tests on various concentrations of the standard heparin used in this laboratory yielded results within  $\pm 10$  per cent of the theoretic potencies.

## SUMMARY

1. A method for the assay of the anticoagulant activity of heparin using recalcified fresh or frozen beef or sheep plasma has been presented.
2. The end point is definite.
3. The method is rapid, simple to execute, reproducible within less than 10 per cent error, employs simple calculation for potency determination, and enables the repetition of the assay using the same material under similar conditions of assay.

## REFERENCES

1. Foster, R. H. K.: Assay of Heparin, *J. LAB. & CLIN. MED.* 27: S20, 1942.
2. Charles, A. F., and Scott, D. A.: Studies on Heparin, *Biochem. J.* 30: 1927, 1936.
3. Jacques, L. B., and Charles, A. F.: The Assay of Heparin, *Quart. J. Pharm. & Pharmacol.* 14: 1, 1941.
4. Fischer, A., and Schmitz, A.: On the Chemical Nature of Heparin. I. The Quantitative Determination of Heparin, *Ztschr. f. physiol. Chem.* 210: 129, 1932.
5. Kuizenga, M. H., Nelson, J. W., and Cartland, G. F.: The Bioassay of Heparin, *Am. J. Physiol.* 139: 612, 1943.
6. Taylor, F., Lozner, E., Davidson, C., Tagnon, H., and Newhouser, L.: Preservation of Normal Human Plasma in the Liquid State. II. Comparative In-vitro Studies on the Physiologic Activity of Labile Constituents of Liquid and Frozen Plasma, *J. Clin. Investigation* 23: 351, 1944.
7. Reinert, M., and Winterstein, A.: Contribution to the Study of Heparin, *Arch. internat. de pharmacodyn. et de therap.* 62: 47, 1939.

## PERMANENT METACHROMATIC STAINING OF MUCUS IN TISSUE SECTIONS AND SMEARS

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THE cytology of mucous secretion in the digestive tract has been neglected as a field of investigation in gastroenterology, in part because of the lack of a satisfactory staining method for the mucins. The only stain for this purpose which has withstood the test of time is mucicarmine, and this has been reliable only for mucus in typical goblet cells. In the stomach, where the mucus normally occurs in the columnar surface epithelium and the neck chief cells, its reliability is notably poor. Thionine and toluidine blue are examples of another type of mucin stain. These dyes differentiate sulfuric acid esters of high molecular weight (for example, the mucins) from other tissue components by a characteristic bichromatic effect called metachromasia, and thereby obviate the need for counterstaining for this purpose. In the case of toluidine blue the mucins are stained some shade of red or purple, whereas other cellular components are stained blue. In addition to this advantage, the metachromatic stains are superior to mucicarmine in respect to their sensitivity and specificity. For these reasons, toluidine blue or thionine would long ago have become the preferred mucin stain were it not for the fact that their characteristic bichromatic effect is lost as soon as the stained tissue is subjected to any dehydrating procedure.

Some time ago we<sup>3</sup> reported a technique for permanent metachromatic staining which was suitable for smears of viscous gastric mucus but not for tissue sections. When this method was applied to smears of secretion from gastric pouches stimulated with mustard oil, or to smears of human gastric juice, it often resulted in the loss of most of the material during the staining process. Although tissue sections were stained effectively, the metachromatic effect was transient. Furthermore, smears treated in this way frequently showed disintegration of the cytoplasmic portion of the cells, but the nuclei were well preserved.

The present investigation was undertaken in an effort to overcome these major deficiencies. To this end, it was necessary to reconsider every step in the procedure and to test it repeatedly on a variety of tissues from animals of different species, as well as on mucus from dog and man.

*Fixation.*—Smears of mucous material gave excellent results after simple air drying, without chemical fixation. For tissues, a number of fixatives were tried without success. Ten per cent formalin failed to preserve the mucus effectively and stained connective tissue red as well. After fixing with Helly's

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fluid the tissue took the dye, but poorly. A saturated solution of mercuric chloride led to considerable shrinkage and caused precipitation of the dye. A saturated solution of mercuric chloride containing 5 per cent acetic acid gave irregular, granular staining. Alcohol-formol or 70 per cent alcohol alone prevented staining of mucinous substances altogether. The one fixative which gave truly satisfactory results was Zenker's solution with acetic acid, prepared without the addition of sodium sulfate. Neither potassium bichromate nor mercuric chloride alone yielded the same degree of color intensity and brilliance as a mixture of the two. This fixative preserved both intra- and extracellular mucus and augmented the contrast between the metachromatic and normochromatic structures without inducing a diffuse metachromatic staining of the connective tissue. Another advantage was preservation of cytologic detail. Toluidine blue is in general an excellent nuclear stain, but its efficiency in this regard is markedly increased by Zenker's fixation.

*Staining.*—After repeated trials under a variety of conditions, the staining solution finally adopted contained 0.25 Gm. of toluidine blue per 100 ml. of 0.25 per cent borax solution. When the dye was more dilute than this, it did not stain deeply enough, whereas more concentrated solutions led to a diffuse purple staining of all structures with practically no bichromatic differentiation. The pH of the staining solution is fixed about 9.0 by the borax. It was found that most of the tissues could be stained well also with a simple aqueous solution of the dye (pH 5.5), except for the surface epithelium of feline and human stomachs. In these two instances, the intracellular mucus failed to take the dye unless the pH of the staining solution was adjusted to 9.0, a value which was found to be adequate for all the other tissues and all the smears. After the usual processing of the paraffin sections from xylol to water, it was found desirable to rinse the slides in a fluid of about the same pH (for example, 0.25 per cent borax) both before and after staining. Smears are rinsed only after being stained. The optimal staining time was thirty seconds for all materials examined. Any prolongation beyond this limit leads to overstaining and a nonspecific purple coloration. There is a tendency for the connective tissue to be stained metachromatically at this stage, but this usually disappears during subsequent treatment.

*Preservation of Metachromasia.*—Ever since the investigations of Hoyer<sup>5</sup> it has been known that the metachromatic effect disappears as soon as the stained sections are treated with alcohol. Attempts have been made to overcome this difficulty by mounting the water-wet preparation directly in a glycerine-containing medium or in a levulose syrup<sup>9</sup>; also, by dehydrating with acetone instead of alcohol<sup>7</sup> before treatment with xylol for mounting in balsam. All of these techniques failed to give us the desired permanence, as did a multitude of other empiric procedures. It was finally found that metachromasia can be preserved if the stained and washed section is treated with an aqueous solution of 0.5 per cent potassium bichromate, saturated at room temperature with mercuric chloride.

*Dehydration and Mounting.*—Following this treatment with bichromate-sublimate solution, the slide is washed with absolute alcohol. If other than

anhydrous alcohol is used, connective tissue, smooth muscle fibers, and even some nuclei acquire a red coloration. It also happens that an occasional finished preparation shows streaks of blue dye. Both of these irregularities can be prevented if each slide is rinsed thoroughly with a stream of fresh absolute alcohol from a siphon bottle, instead of being immersed in a staining dish containing alcohol which has become diluted from repeated use. Omission of the usual treatment with graded concentrations of alcohol does not lead to any considerable shrinkage. At this stage of the procedure the section shows excellent metachromasia, but when it is brought into xylol all its elements acquire a bluish coloration. With this reduction in reddish tones the normochromatic elements become a purer blue, but there is a loss in sharpness of the metachromatic differentiation. Such purification of color tone in the normochromatic elements is distinctly advantageous, and it can be retained even when the metachromasia is reintensified by the next step; that is, retreatment with absolute alcohol followed by the bichromate-sublimate mixture. The section is then washed with absolute alcohol containing 1 per cent of rosin, which intensifies the red shades with toluidine blue as it has been shown to do with Giemsa stain. The slide is then carried through two changes of benzol and mounted in Canada balsam dissolved in benzol. The use of xylol, toluol, or acetone for clearing or as solvent for the balsam must be avoided because they destroy the metachromasia. Clarite may be substituted for benzol-balsam, but the results are less satisfactory.

#### OUTLINE OF THE STAINING TECHNIQUE

Tissues are fixed in Zenker's solution containing 5 per cent acetic acid, and the paraffin sections are carried through to water in the usual way. They are then rinsed in 0.25 per cent aqueous borax solution and drained free of surplus fluid. Smears are prepared from mucus specimens immediately after collection and are left to dry at room temperature for the *minimum* time required. This is usually two to four hours. Mucus smears are not given the preliminary rinsing in borax solution. Slides of both smears and tissue sections are then treated as follows:

1. Cover for thirty seconds with ample staining solution (0.25 Gm. toluidine blue 0\* dissolved in 100 ml. of 0.25 per cent borax solution).
2. Rinse with 0.25 per cent borax solution.
3. Cover for fifteen seconds with a 0.5 per cent solution of potassium bichromate, which has been saturated with mercuric chloride at room temperature and filtered, and drain.
4. Cover the slide for two minutes with a fresh portion of the bichromate-sublimate solution and drain.
5. Wash thoroughly for about ten seconds with a stream of fresh absolute alcohol from a siphon bottle.
6. Immerse in xylol for thirty seconds.
7. Rinse again with absolute alcohol and drain completely.

\*National Aniline, Certified.

8. Cover again with the bichromate-sublimate solution for thirty seconds.
9. Rinse thoroughly with rosin alcohol (1 Gm. rosin, U. S. P., dissolved in 100 ml. absolute alcohol) and drain.
10. Carry through two changes of pure benzol (one minute each).
11. Mount in Canada balsam dissolved in benzol.

#### DISCUSSION

In order to assure ourselves of the permanence of the metachromatic differentiation obtained by means of the foregoing procedure, it was applied to a variety of tissues in May, 1945. For this purpose specimens from stomach, jejunum, colon, salivary glands, and bronchi were obtained from the dog, cat, rat, guinea pig, and rabbit. Specimens of resected human stomachs, taken at operation, and guinea pig cervix were also included, as were smears of mucus collected from Heidenhain pouch dogs and human stomachs under eugenol stimulation.<sup>4</sup> At the time of this report, two years after preparation, these slides continue to show excellent preservation of metachromatic as well as normochromatic colors. Only in the goblet cells of rat jejunum is there any evidence of a loss of metachromasia, but even in this instance some areas of the section still contain purple-stained material.

Various theories have been advanced to account for the metachromatic properties of certain dyes like toluidine blue.<sup>1-3, 12, 6, 8</sup> Recently, Michaelis and Granick<sup>9</sup> added quantitative spectrophotometric evidence to support the view that the shift from blue to purple is due to polymerization of the dye molecules. The simple monomeric form possesses the normal color (pure blue), whereas the polymers are purple to pink, depending on the degree of polymerization. Increasing the concentration of dye induces a shift in color toward the purple, even in the absence of a metachromatically staining substance,<sup>10</sup> and this change is completely reversible on subsequent dilution. This effect is readily explained by the polymerization theory. Alcoholic solutions of the dye show only the blue coloration, and treatment of metachromatically stained substances with alcohol invariably leads to a disappearance of metachromasia. This reversal can be prevented by the action of a mixture of potassium bichromate and mercuric chloride on the metachromatic complex of dye and substrate. As yet, no explanation has been offered for either the deleterious effect of alcohol on metachromasia or the stabilizing effect of the bichromate-sublimate mixture. It is interesting, however, that treatment with the latter does not induce metachromatic staining of previously normochromatic structures; it merely preserves and improves the effects which are already existent.

The superiority of the present technique over that previously reported by us may be ascribed to the change in pH of the staining and washing fluids from an acid value (circa 3) to an alkaline one (circa 9). As a result, cytoplasmic destruction in smears is eliminated and loss of material from the slide is prevented. Although the success of this method in tissue sections depends largely upon this different pH, it is the use of the bichromate-sublimate mixture which effects the permanence of the metachromasia. On the other hand, the present



procedure possesses certain obvious disadvantages. Occasionally the finished preparations of smears, but not of paraffin sections, contain residues of bichromate which give a disturbing yellow-green coloration in some areas, and as yet we have not been able to eliminate this. Also, the method is applicable only to tissues which have been fixed in Zenker's solution; if it could be used after formalin and Bouin's fluid, this would be a decided advantage for the purposes of the routine pathology laboratory. We hope that these difficulties may also be overcome in time and that the entire procedure may be simplified.

We wish to express our thanks to Dr. B. P. Sonnenblick, who stained a great variety of tissue sections and smears by this method in order to confirm our experience with it.

## REFERENCES

1. Bank, O., and Bungenberg de Jong, H. G.: Untersuchungen ueber Metachromasie, *Protoplasma* 32: 489, 1939.
2. Hempelmann, Jr., L. H.: Staining Reactions of the Mucoproteins, *Anat. Rec.* 78: 197, 1940.
3. Hess, M., and Hollander, F.: Permanent Metachromatic Staining of Gastric Mucus Smears, *J. LAB. & CLIN. MED.* 29: 321, 449, 1944.
4. Hollander, F., and Lauber, F. U.: Comparison of Eugenol With Other Stimuli for Gastric Mucus Secretion, Communications XVII International Physiological Congress, 1947.
- 4a. Holmes, W. C.: The Chemical Nature of Metachromasy, *Stain Technol.* 1: 116, 1926.
5. Hoyer, H.: Ueber den Nachweis des Mucins in Geweben mittels der Faerbemethode, *Arch. f. mikr. Anat. Bonn.* 36: 310, 1890.
6. Kelley, E. G., and Miller, Jr., E. G.: Reactions of Dyes With Cell Substances. II. The Differential Staining of Nucleo-Protein and Mucin by Thionine and Similar Dyes, *J. Biol. Chem.* 110: 119, 1935.
7. Lillie, R. D.: A Brief Method for the Demonstration of Mucin, *J. Tech. Methods* 12: 120, 1929.
8. Lison, L.: Études sur la metachromasie. Colorants metachromatiques et substances chromotropes, *Arch. de biol., Paris* 46: 599, 1935.
9. Michaelis, L., and Granick, S.: Metachromasy of Basic Dyestuffs, *J. Am. Chem. Soc.* 67: 1212-19, 1945.
10. Moellendorff, W., and Moellendorff, M.: Durchtraenkungs und Niederschlagsfaerbung als Haupteerscheinung bei der histologischen Faerbung, *Ergebn. d. Anat. u. Entwicklungsgesch., Wiesb.* 25: 1, 1924.

## RETICULUM STAINING WITH SCHIFF REAGENT AFTER OXIDATION BY ACIDIFIED SODIUM PERIODATE

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THE method to be reported herein was evolved from studies on the relation of the Bauer<sup>1</sup> glycogen method to the Jackson-Hudson<sup>2</sup> periodate oxidation of polysaccharides. These studies are being published elsewhere in extenso.

Glycogen, epithelial mucin, cellulose, starch, yeast, and mold chitin and cartilage matrix are stained purplish-red by the Bauer technique.<sup>3</sup> When an acid sodium periodate solution is substituted for the chromic acid of the Bauer method, reticulum of certain parenchymatous organs, notably lung, kidney, thyroid, and cerebral capillaries, is stained a brilliant purplish-red; collagen and fibrin, pink. Capsular substance of certain bacteria and certain elements in Actinomyces granules also stains pink, but further detailed studies are necessary on this.

The technique employed is as follows:

1. Thin ( $5\ \mu$  or less) paraffin sections of routine material fixed in formalin, Orth's fluid, or other fixatives are deparaffinized and brought to 100 per cent alcohol as usual.
2. Soak five minutes in 1 per cent collodion in 50:50 ether and 100 per cent alcohol mixture. Drain one minute with slides held vertically, and harden five minutes, or as much longer as convenient, in 80 per cent alcohol.
3. Wash in water and immerse for ten minutes in sodium periodate ( $\text{Na}_2\text{IO}_5$ ), 1 Gm.; 70 per cent nitric acid (specific gravity, 1.42), 0.5 c.c.; distilled water, 100 cubic centimeters.
4. Wash five minutes in running water.
5. Immerse for fifteen minutes in Schiff reagent,<sup>†</sup> agitating at three- to five-minute intervals, preferably by pouring the fluid out and back.
6. Transfer directly to three successive baths of one and one-half minutes each in 0.5 per cent sodium bisulfite ( $\text{NaHSO}_3$ ). (This is slightly weaker than Bensley's<sup>4</sup> M/20.)

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<sup>†</sup>The Schiff reagent is made as follows: Dissolve 1 Gm. basic fuchsin in 100 c.c. distilled water at 90 to 95° C.; filter while cooling at about 60 to 70° C. When cool, add 2 Gm. sodium bisulfite ( $\text{NaHSO}_3$ ) and 20 c.c. normal hydrochloric acid; stopper tightly; put away in cool, dark place overnight. In the morning the solution should be somewhat brownish-yellow in color. Add 300 mg. of finely ground charcoal, shake for one minute, and filter. The solution should become pale yellow. Store in refrigerator at 0 to 5° C. The solution may be used for several weeks, at least if kept cold. Discard if a pink color appears.

7. Wash ten minutes in running water.
8. Stain five to six minutes in Weigert's<sup>1</sup> acid iron chloride hematoxylin. Wash in water.
9. Counterstain one minute in saturated aqueous picric acid solution or in 1 per cent orange G.
10. Dehydrate with alcohols and clear in one change of xylene and 100 per cent alcohol mixture (50:50) and two changes of xylene; mount in clarite.

The Weigert fibrin routine may be introduced in place of steps 9 and 10.

- a. Stain in Conn's<sup>6</sup> ammonium oxalate crystal violet (or any other aqueous or hydroalcoholic crystal violet) for two or more minutes.
- b. Wash off quickly with 0.9 per cent sodium chloride solution.
- c. Treat with the Weigert-Lugol<sup>7</sup> iodine solution (1 Gm. iodine, 2 Gm. potassium iodide, 100 ml. distilled water) for thirty seconds.
- d. Blot dry with hard filter paper and decolorize with several successive changes of a few drops each of aniline and xylene (50:50) mixture until violet color ceases to come out.
- e. Wash in two or more changes in xylene; mount in clarite.

#### RESULTS

By all three variants discussed, pulmonary reticulum, renal reticulum, and glomerular stroma, the reticulum of intestinal smooth muscle, the subepithelial basement membrane, the supporting stroma of the cerebral capillaries, and the reticulum of the gastric mucosa were stained a bright purplish-red. Collagen stains pink. Fibrin, hyaline droplets in renal epithelium, hyaline casts, and serous coagula in pulmonary alveoli also stain dull pink of a variable intensity when no plasma stain or the picric acid or orange G variants are used. With the Weigert fibrin variant, fibrin, hyaline droplets, and serous coagula vary from pink to violet, and red corpuseles stain gray to blue-violet. Orange G tinges the erythrocytes orange-yellow but has little other evident effect. Picric acid gives bright yellow erythrocytes and yellow to brown cytoplasm, and gives a yellow overcast to the pink of fibrin, serum, and the like.

With all variants, cartilage matrix is stained red-purple; epithelial mucin, reddish-purple to almost violet; glycogen, dark red-purple to almost black. If confusing, the glycogen may be removed by interposing the Lillie-Greco<sup>3</sup> diastase digestion technique between Steps 1 and 2.

McManus<sup>8</sup> reported the use of a periodic acid Schiff technique for the demonstration of mucin, and recently presented the extension of this technique to the demonstration of renal capillary stroma.\*

\*At the American Association of Pathologists and Bacteriologists, May 17, 1947, Chicago, Ill.

## REFERENCES

1. Bauer, H.: Mikroskopisch chemischer Nachweis von Glykogen und einigen anderen Polysacchariden, *Ztschr. f. wissenschaft. mikr. anat. Forsch.* 33: 143-160, 1933.
2. Jackson, E. L., and Hudson, C. S.: Application of the Cleavage Type of Oxidation by Periodic Acid to Starch and Cellulose, *J. Am. Chem. Soc.* 59: 2019-20, 1937.
3. Lillie, R. D.: Reactions of Various Parasitic Organisms in Tissues to the Bauer, Feulgen, Gram and Gram-Weigert Methods, *J. Lab. & Clin. Med.* 32: 76-88, 1947.
4. Bensley, Caroline M.: Comparison of Methods for Demonstrating Glycogen Microscopically, *Stain Technol.* 14: 17-22, 1939.
5. Weigert, C.: Eine kleine Verbesserung der Hamatoxylin-van Gieson-Methode, *Ztschr. f. wissenschaft. mikr.* 21: 1, 1904.
6. Conn, H. J., and Darrow, M. A.: *Staining Procedures, 1911-1944*, Geneva, N. Y., Biotech.
7. Ehrlich, P.: *Encyklopadie der mikroskopischen Technik*, Berlin and Wien, 1903, Urban & Schwarzenberg.
8. Lillie, R. D., and Greco, J.: Malt Diastase and Ptyalin in Place of Saliva in the Identification of Glycogen, *Stain Technol.* 22: 67-70, 1947.
9. McManus, J. F. A.: *The Histological Demonstration of Mucin After Periodic Acid*, Nature, London 158: 202, 1946.

## THE QUANTITATIVE DETERMINATION OF AMPHETAMINE

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A SATISFACTORY and shortened method for the quantitative recovery of amphetamine from human organs was evolved following repeated experiments *in vitro*.

Because of the ever-widening use of beta-phenylisopropylamine, variously sold under the name of Amphetamine, Benzedrine, Elastonon, Isoamin, Neco-drin, and Orledrin, a study was undertaken to obtain an efficient method of quantitative determination of amphetamine. A technique of extraction is described, using a sulfuric-tungstic acid filtrate, as well as a method of quantitative determination; these greatly reduce the time element in the separation of amphetamine from human organs.

We have used a new technique of approach, employing a combined method of purification, distillation, and diazotization, which is presented in detail.

### METHOD

To 25 Gm. of blood, urine, or finely minced organs in a 1 liter flask, add 150 ml. of distilled water. Shake well for five minutes and allow to stand for ten minutes longer. Add 10 ml. of 10 per cent NaOH and shake well for five minutes; then add 30 ml. of 10 per cent sodium tungstate and shake well. Next add slowly 30 ml. of 1 N-H<sub>2</sub>SO<sub>4</sub> with continuous shaking, and finally acidify with 18 N H<sub>2</sub>SO<sub>4</sub> and add 5 ml. in excess. Allow to stand for fifteen minutes and filter through a No. 12 Whatman folded filter paper. Transfer the filtrate to a 1 liter round bottomed flask and make neutral with 10 per cent NaOH; then add 1 ml. in excess. Steam distill through a short neck flask connected to a Liebig condenser and collect 200 ml. of distillate in a flask containing 10 ml. of 4 N H<sub>2</sub>SO<sub>4</sub>. Transfer the distillate to a 500 ml. extraction flask, add 20 ml. of ether and make alkaline with 10 per cent NaOH and add 1 ml. in excess. Shake the flask for one minute and extract with three more portions of ether. Combine the ether extracts, wash with water, and discard the water. Extract the ether layer with three 10 ml. portions of 0.5 per cent HCl. Evaporate the HCl extract to dryness over steam bath and diazotize as follows:

Take up residue with 1 ml. of water and add 5 ml. of cold diazotizing reagent (keep volume below 2 ml. for a larger volume of water causes a reduction of color due to failure of complete reaction with diazotizing reagents). Then add 5 ml. of 1.1 per cent sodium carbonate drop by drop with continuous mixing and allow to stand for fifteen minutes. Next add 1 ml. of 10 per cent NaOH drop by drop with continuous mixing and allow to stand for ten minutes. Dilute with water to 40 ml. and mix. Add 10 ml. of n butanol and mix by inverting. After colored layer has cleared, read transmission in spectrophotometer at 530 mμ and calculate the amount of amphetamine present from a standard curve.

Nicotine and pyridine do not interfere with the above separation. The amphetamine is identified in an aliquot part of the distillate by obtaining the melting point of the picrate (142° to 143° C.) and the picrolonate (195° to 196° C.)

REAGENTS<sup>1</sup>

## 1. p-Nitroaniline Hydrochloride

To 0.69 Gm. p-nitroaniline in a 125 ml. glass-stoppered Erlenmeyer flask, add 3 ml. of concentrated HCl. Mix by twirling and break larger lumps with a glass rod. Let stand for ten minutes from the time of addition of the acid. Add 97 ml. of distilled water, a little at a time, with continuous mixing. Stopper the flask and shake vigorously. Filter on a 9 cm. Whatman No. 4 paper, collecting filtrate in a 125 ml. glass-stoppered Pyrex bottle. This solution should be stable at room temperature for at least a year.

2. 0.7 Per Cent Sodium Nitrite ( $\text{NaNO}_2$ )

Dissolve 0.7 Gm. reagent grade  $\text{NaNO}_2$  in distilled water and dilute to 100 milliliters. This solution should be refrigerated at all times and should not be used after it is more than one week old.

## 3. Diazo Reagent (p-Nitrobenzenediazonium Chloride Solution)

To 5 ml. p-nitroaniline hydrochloride in a 100 ml. glass-stoppered volumetric flask, add 1 ml. of concentrated HCl. Place the flask in an ice-salt bath for ten minutes and then add 3 ml. of 0.7 per cent solution of sodium nitrite. Let stand in ice-salt bath for six minutes, dilute to volume with distilled water (room temperature), mix, and return to ice salt bath for ten minutes. The reagent is now ready for use and will keep at 0 to 1° C. for at least two weeks under these conditions. It should be tested with amphetamine sulfate standard before using it with unknowns.

4. 1.1 Per Cent Sodium Carbonate ( $\text{Na}_2\text{CO}_3$ ) Anhydrous

Dissolve 2.75 Gm.  $\text{Na}_2\text{CO}_3$  (reagent grade) in distilled water and dilute to 250 ml. in a Pyrex bottle.

## 5. 10 Per Cent Sodium Hydroxide

Made to contain 10 Gm. NaOH in 100 ml. solution; store in a rubber-stoppered 250 ml. Pyrex bottle.

## 6. n-Butanol

## 7. Standard Amphetamine Sulfate\*

Made to contain 0.03 mg. amphetamine sulfate per cubic centimeter.

Beyer and Skinner<sup>1</sup> described a new method for amphetamine determination in urine. By diazotizing the extracted amphetamine with p-nitrobenzene, they obtained a red color which was intensified by shaking out with n-butanol.

Gad<sup>2</sup> described a distillation method for tissues. His distillate was collected in 25 ml. of 4 N  $\text{H}_2\text{SO}_4$ . The distillate was made alkaline, then extracted with benzol, and the benzol portion then extracted with 0.02 N HCl. The unused portion of the acid was titrated with 0.02 N sodium hydroxide using methyl red as indicator. Gad also used the spectrophotometer in estimating the amount of amphetamine. The objection to his method was the length of time required and consequent tediousness, as a result of its troublesome foaming.

Using Gad's method, we found that post-mortem material, amphetamine-free, educed a color reaction when tested by the Beyer and Skinner method. We then subjected our distillate, using a sulfuric-tungstic acid filtrate, to the Beyer and Skinner method of estimation of amphetamine, and the result was a colorless blank. With known quantities, a well-defined color was obtained, which could be compared with suitable standards.

Table I is presented to demonstrate that amphetamine is quantitatively recoverable from the liver.

\*Smith, Kline and French Laboratories.

TABLE I. RECOVERY OF ADDED AMPHETAMINE SULFATE FROM 25 GM. OF LIVER

EXP.	NaOH CONC.	MG. OF AMPHET- AMINE SULFATE ADDED	ML. DISTILLED	MG. RECOVERED	% RECOVERY
1	10%	1.0	250	0.20	20
2	5%	1.0	600	0.43	43
3	1 ml. of 10% NaOH in excess	1.0	100	0.98	98
4	1 ml. of 10% NaOH in excess	2.0	200	1.80	90
5	1 ml. of 10% NaOH in excess	1.0	200	0.95	95
6	1 ml. of 10% NaOH in excess	1.0	200	0.95	95

The precipitated protein residue was tested for the presence of amphetamine with negative results. The distillation was conducted at a pH of 8.

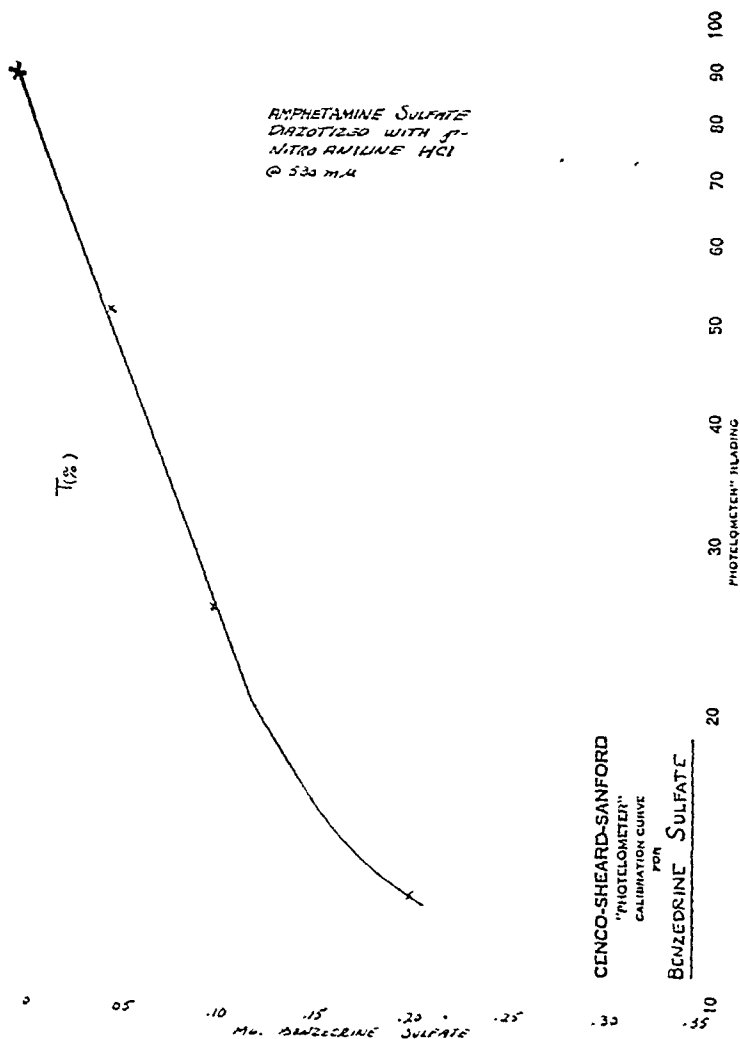


Fig. 1.

Many other investigators have advanced methods of amphetamine distillation. Among them was Schoen,<sup>4</sup> who distilled a tablet into standard sulfuric acid and titrated the unused acid. Keenan<sup>4</sup> described a gold chloride micro-precipitation method. Reznek<sup>5</sup> extracted solutions of amphetamine from alkaline solutions with chloroform. The chloroform was extracted with 0.1 N  $H_2SO_4$ , and the unused acid was titrated with 0.1 N alkali (1 ml. of 0.1 N acid was equal to 0.0135 Gm. of amphetamine). Dultz<sup>6</sup> used a method for detecting beta-phenylisopropylamine, and beta-phenylisopropylmethylamine based on the determination of the melting points of the picrolonates. Cannon<sup>7</sup> described a microchemical test with platinum chloride, a second method using a benzol derivative with a melting point of 134° to 135° C., and a third method based on coupling with p-nitrobenzene diazonium chloride. This same author<sup>8</sup> later described a modified technique for amphetamine determination and reported a collaborative study of previously described methods. Illing<sup>9</sup> reported that the distillation of alkaline mixtures of viscera was practically impossible and that when acidified with acetic acid the distillate gave negative results for amphetamine.

These methods for the most part were found wanting, for our observations, from repeated experiments, indicated that the distillation method alone or the tungstate precipitation without distillation gave interfering color.

Our application of distillation and extraction procedures to aqueous solutions of amphetamine sulfate gave quantitative recoveries. This combined method of purification of organs by precipitation with sulfuric and tungstic acid gave clear solutions which filter rapidly. The method will detect and estimate as low an amount of amphetamine as 0.03 mg. in 25 Gm. of tissue. It can be applied to blood and urine as well as to tissues and does not require a blank to be subtracted.

We found the time for quantitative separation from tissues greatly reduced with this method and found it to be a much shorter procedure than the usual modified Stas-Otto method, used routinely in our laboratory.<sup>10</sup> Another salient feature was the elimination of foaming which was encountered in the Gad distillation.

#### SUMMARY

A new quantitative method for the determination of amphetamine in organs, using sulfuric-tungstic acid filtrate, distilled, is described.

It is shown that quantitative recoveries were obtained from the application of distillation and extraction procedures to aqueous solutions of amphetamine.

This method is applicable to blood and urine as well as to tissues. It will estimate an amount of amphetamine as low as 0.03 mg. in 25 Gm. of tissue.

#### REFERENCES

1. Beyer, K. H., and Skinner, J. T.: The Detoxication and Excretion of Beta-Phenylisopropylamine (Benzedrine), *J. Pharm. & Exper. Therap.* 68: 419-432, 1940.
2. Gad, Inger: Das Verhalten des  $\beta$ -Phenylisopropylamine im Kaninchenorganismus. I. Bestimmung des Gehalts an  $\beta$ -Phenylisopropylamin in Geweben und Organen (The



- Behavior of  $\beta$ -Phenylisopropylamine in the Rabbit. I. Determination of  $\beta$ -Phenylisopropylamine in Tissues and Organs), Arch. f. exper. Path. u. Pharmacol. 196: 25-33, 1940. See also Jacobsen, E., and Gad, I.: Das Verhalten des  $\beta$ -Phenylisopropylamine im Kaninchenorganismus. II. Abbau, Organverteilung und Ausscheidung bei normalen Tieren (The Behavior of  $\beta$ -Phenylisopropylamine in the Rabbit. II. Decomposition, Organ Distribution and Excretion in Normal Animals), Arch. f. exper. Path. u. Pharmacol. 196: 34-42, 1940. Larson, V.: Das Verhalten des  $\beta$ -Phenylisopropylamine in Kaninchenorganismus. III. Der Abbau des  $\beta$ -Phenylisopropylamine in überlebenden Kaninchenorganismus (The Behavior of  $\beta$ -Phenylisopropylamine in the Rabbit. III. The Decomposition of  $\beta$ -Phenylisopropylamine in Isolated Rabbit Organs), Arch. f. exper. Path. u. Pharmacol. 196: 43-50, 1940, Hald, J., and Gad, Inger: I. Om  $\beta$ -Fenylisopropylaminets Fysiske og Kemiske Egenskaber (The Physical and Chemical Properties of  $\beta$ -Phenylisopropylamine), Dansk Tidsskr. farm. 12: 97-104, 1938.
3. Schoen, Karl: A Rapid and Simple Method for the Determination of Ephedrine, J. Am. Pharm. 33: 116, 1944.
  4. Keenan, George L.: Report on Microchemical Tests for Alkaloids and Synthetics, J. A. Off. Agric. Chem. 25: 830, 1942.
  5. Reznak, S.: Determination of Amphetamine (Benzedrine), J. A. Off. Agric. Chem. 25: 746, 1942.
  6. Dultz, G.: Nachweis und Bestimmung von Beta-Phenylisopropylamin und Beta-Phenylisopropylmethyamin (Detection and Estimation of Beta-phenylisopropylamine and Beta-phenylisopropylmethyamine), Ztschr. Anal. Chem. 120: 84, 1940.
  7. Cannon, James H.: Report on Benzedrine, J. A. Off. Agric. Chem. 24: 803, 1941.
  8. Cannon, James H.: Report on Benzedrine, J. A. Off. Agric. Chem. 25: 788, 1942.
  9. Illing, E. T.: Detection and Estimation of Benzedrine, Analyst 65: 3, 1940.
  10. McNally, W. D.: Toxicology 30, 1937.

## A MICROSCREW CLAMP FOR USE WITH MOHR TYPE OF MICROBURETTES

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THE Koch\* microburette, in common use for many years, consists of a small capacity burette, 1 to 10 ml., graduated in 0.01 or 0.05 ml., and connected to a reservoir through a glass stopcock. A troublesome feature inherent with any apparatus having glass stopcocks is the gradual accumulation of particles of grease and a film of grease which prevents wetting of the wall of the graduated tube. This results from gradual loosening of the grease by repeated turning of the stopcock. No lubricant, including silicone, overcomes this difficulty. Frequent treatment with cleaning solution is necessary and involves a lot of time and labor. With heparin assays, acid cleaning after every assay is found necessary to give a good meniscus. Another disadvantage is that after several manipulations the accumulation of grease on the margin of the stopcock bore forms a sort of flap valve which makes accurate control impossible. Control of the flow also becomes difficult as the lubricant becomes thinned out and emulsified with consequent sticking.

To obviate these difficulties a microscrew clamp to use with rubber tubing has been designed. The first consideration was to provide a thumbscrew that would work easily yet without play. Second, a shut-off stop was needed to limit the compression of the rubber tubing just enough to stop the flow. Third, an opening stop seemed desirable to permit instant opening to a predetermined degree. This would regulate the maximum rate of outflow from the burette. The main features of the clamp will be described although Fig. 1 is self-explanatory. A photograph of the clamp is shown in Fig. 2.

The screw clamp shown is designed to take a  $\frac{3}{16}$  inch outside diameter pure gum tube, but the clamp may be made for any size. The rubber tube is maintained in position by four pins. A U-shaped clamp, shown in the lower part of the front view and in section A-A, is fitted over the lower end of the rubber tube in which is inserted the glass delivery tip and holds the latter steady.

The clamp is held together by two threaded rods, an extension of one serving as the stop. The compression bar is slightly rounded on its contact surface. A strong bronze spring forces the compression bar assembly firmly against the lower end of the thumbscrew through a steel ball bearing. Play is thus eliminated and friction in the step bearing minimized. The thumbscrew turns in a threaded bronze bushing, and the large knurled knob provides for fine control

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These microscrew clamps may be obtained from Earl L. Page, Nashville, Ind.

\*Koch, F. C.: Two Convenient Forms of Apparatus for Microblood and Microurine Analysis, J. LAB. & CLIN. MED. 11: 774, 1926.



It has been found that this microscrew clamp offers an ease of manipulation quite outside the range provided by glass stopcocks. In heparin assays several hundred portions of solutions have been dispensed from a series of microburettes. One's fingers become tired in adjusting the glass stopcocks, even to the point of being unable to control accurately a stopcock still turning smoothly. This microscrew clamp virtually eliminated fatigue in the experiments run, and the speed of operation was increased. Volumes as small as 0.001 ml. may be dispensed if the burette bore is small enough. A microburette with this specially designed screw clamp should be useful not only in heparin assays, but also in many types of work where small volumes are to be dispensed, or for microtitrations.

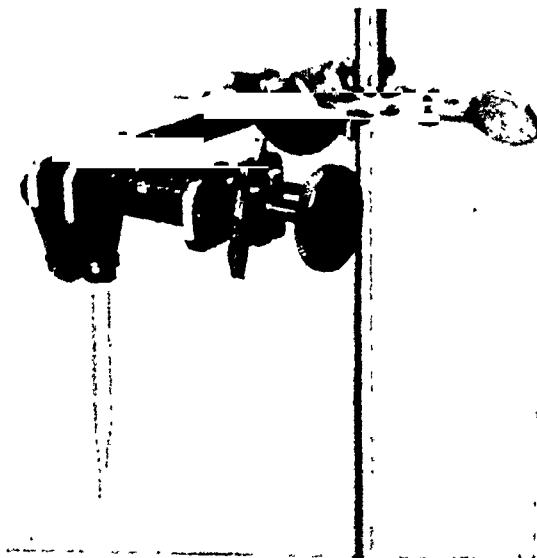


Fig. 2.

#### SUMMARY

A microscrew clamp has been described for use with microburettes which offers the following advantages over glass stopcocks:

1. Minute control to 0.001 ml. is possible.
2. Faster operation is attained through the use of adjustable stops for the open and closed positions.
3. Faster operation is also attained by the greater ease of manipulation which also reduces fatigue.
4. The frequency of the troublesome acid cleaning is reduced by the elimination of the glass stopcock; and because there is no lubricant to collect on the glass walls, cleaning time itself is reduced.

# AN INSTRUMENT FOR RECORDING MULTIPLE PRESSURES IN MAN

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*Introduction.*—The instrument to be described was developed in order to record multiple pressure changes used in study of the cardiovascular system. These are intra-arterial pressure, pneumatic cuff pulsations and pressure, finger plethysmograph, venous pressure, and respiration. This instrument does not represent an original development as to principle, but it is felt that the modifications and adaptations of previously published techniques have proved to be of sufficient practical value to be described. It was also felt that the method of optical recording with high frequency manometers described by Hamilton and co-workers,<sup>1</sup> and subsequently modified for special purposes by various authors (Green,<sup>2</sup> Gregg and associates,<sup>3</sup> Kotte and associates,<sup>4</sup> and Cahoon and co-workers,<sup>5</sup> was the simplest and most accurate available. However, the relatively long optical levers proved to be too cumbersome for general clinical use in a hospital, where it was desirable to have an instrument that could be transported from one ward to another and used in well-lighted rooms. Portability coupled with the necessity for recording four to six pressures simultaneously required considerable modification of the usual optical manometers.

*General Description (Fig. 1).*—The present instrument consists of three separate units (Fig. 1). One (A, Fig. 1) houses the light source, transformer, manometers and holders, and timing device. It measures 81.28 cm. (32 inches) in length, 38.10 cm. (15 inches) in width, and 35.56 cm. (14 inches) in height. The second unit (B, Fig. 1) is the photokymograph or camera, the base of which is 21.59 cm. (8½ inches) square and the height, 30.48 cm. (12 inches). This construction enables the camera to be used for other purposes. The third unit (CPS, Fig. 1) contains a compressed air tank, pressure indicators, and control valves for inflation and deflation of a pneumatic cuff.

*Optical System (Fig. 2).*—The optical system employed resembles in principle those previously described in that it consists of a light source, the beam of which is reflected from a small mirror mounted on an elastic membrane to moving photographic film (Fig. 2). It is so arranged that the optical length from the mirror to the film is 80 centimeters. The light source consists of a battery of inverted "V" filament 6-volt bulbs arranged to give a vertical filament image. The lights are enclosed in a small plywood housing and require no special ventilation. A cylindric lens which corrects the image of the lamp filament in the vertical axis is placed in front of each manometer. Mounted in the camera is a cylindric lens which corrects the image of the lamp filament in a horizontal axis, thus producing a square or rectangular spot on the film.

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*Timing Device.*—The timing device, giving transverse lines at 0.10 second intervals, consists of a lamp with a rotating slotted disk between it and the camera. The disk is driven by a synchronous motor.

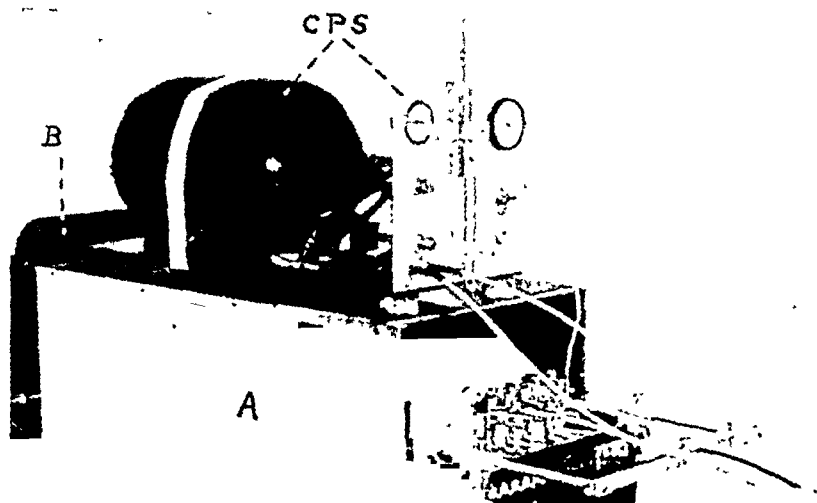


Fig. 1

Fig. 1.—General assembly. A, Housing for light source, transformer, manometers and holders, and timing device; B, camera; CPS, compressed air tank, pressure indicators, and control valves for inflation and deflation of cuff.

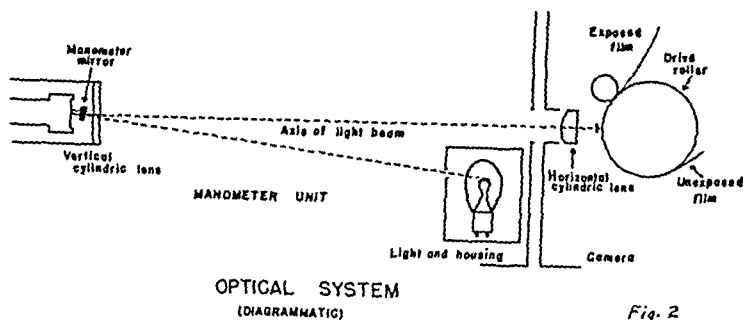


Fig. 2

Fig. 2.

*Manometers.*—The diameter and tension of the manometer membranes vary with the type of pressure to be recorded. Latex rubber is used. For the lowest pressure range (plethysmograph), a large slack manometer membrane (diameter, 2.5 cm.) is used. For intra-arterial pressure recordings, which are in the highest pressure range, tense membrane manometers of 0.5 to 1 cm. in diameter are used. Recently, beryllium copper has become available again and is replacing the latex membranes in higher pressure ranges.

*Manometer Holders (Fig. 3).*—Each manometer assembly is mounted on a small platform on which it can be rotated around a vertical axis so as to place

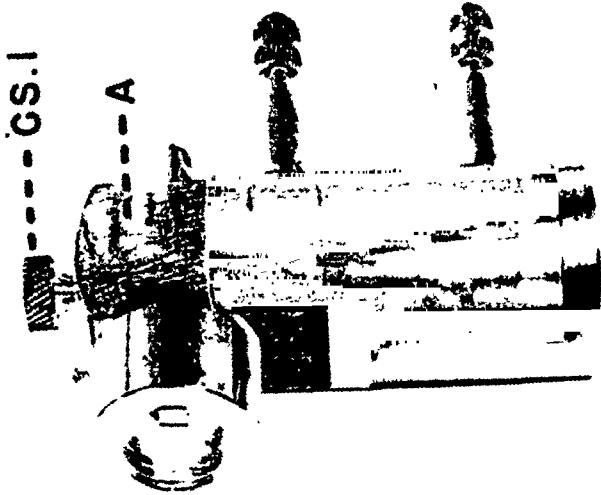
the reflected light beam at any position along the camera slot. This position is maintained by a single clamping screw. The block (A, Fig. 3, Part C) containing the manometer tube rotates on an axis parallel (around pin P, Fig. 3, Part C) to the camera slot and may be clamped in position by a single screw. This movement serves to elevate or depress the reflected light beam in relation to the camera slot. The manometer tube is inserted into a hole in this block so that it may be rotated 360 degrees around a horizontal axis perpendicular to the camera slot and may be clamped in any position by a single screw. This allows the axis of deflection of the light beam to be made parallel to the camera slot (horizontal). The assembled manometers are shown in Fig. 3, Part D.

*Tubing.*—The tubing used to connect the intravascular needles with the manometer should be relatively nonexpansile lest serious damping of the system occur. Polyethylene plastic tubing (I.D.,  $\pm 1$  mm.; wall,  $\pm 2$  mm.) has been found to have several distinct advantages over lead, tin, or zinc tubing. It is chemically inert to the solutions that it contains and therefore forms no precipitate, and it is transparent so that air bubbles may be readily observed. Its flexibility makes it easy to handle.

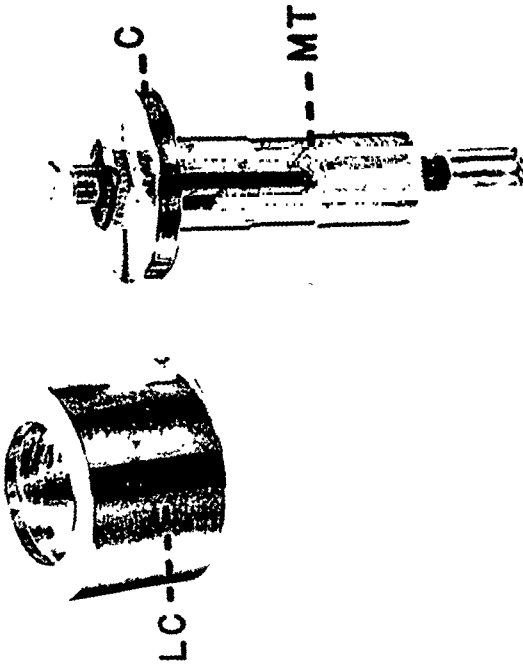
With the advent of small diameter thin-walled polyvinyl tubing which may be passed through 17- or 15-gauge needles, the possibility of utilizing this material and discarding the rigid indwelling needles has been considered. A flexible tube which is relatively inelastic and has a large inside diameter as compared with the outside diameter has many theoretical advantages for intravascular studies. In addition the clotting time of blood in such a tube is greatly prolonged as compared with that in a steel needle tube. For veins, a 17-gauge needle is introduced into the vein and a length of polyvinyl tubing inserted through the needle within the vessel for several centimeters (or any desired length); the needle is then withdrawn, leaving the tubing within the vessel. It is then connected to a manometer by a suitable adapter and flexible tubing. Little bleeding takes place around the indwelling tube.

*Finger Plethysmograph.*—The usual water-filled glass tube plethysmograph has been replaced by an air-filled thin-walled aluminum tube 12 cm. (4.72 inches) in length and 3.5 cm. (1.38 inches) in diameter, which is equipped with a screw-on collar that allows cones with openings of various diameters to be fastened to the tube. The plethysmograph tube with cone in place is slipped over the finger and one of the quick-drying liquid plastics is applied. By this method only relative changes in volume which take place over short periods of time such as with acute venous occlusion (finger blood flow) may be measured.

*Differential Chamber (Fig. 4).*—To record the arterial pressure pulses transmitted through a pneumatic cuff, a manometer must be able to withstand cuff pressures up to 300 mm. Hg or more, yet be responsive to pressure changes of 1 to 5 mm. Hg. The differential capsule principle has been utilized and a small chamber which fits in the described manometer holders has been developed. The membrane holder is screwed into the manometer tube in the usual fashion. The lens cap, with the lens sealed in place, is then placed over the membrane holder and screwed to the manometer tube. Thus a pressure-tight chamber is



B



A



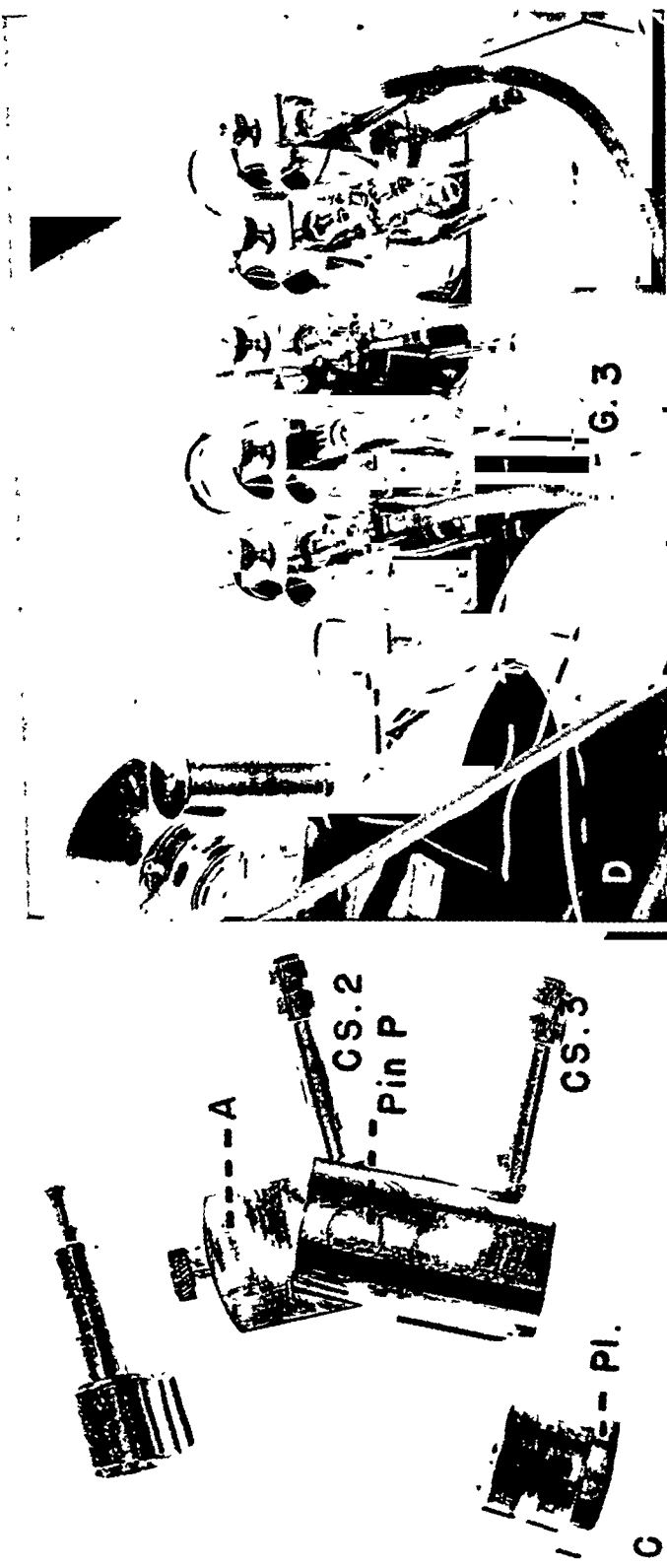


Fig. 3.—Manometer details. Part A, Manometer. MT, Manometer tube; C, collar; LC, cap containing cylindrical lens which is fitted to collar of the manometer.

Part B, Manometer assembly. A, Manometer block. This block contains a circular opening into which the manometer tube fits and allows for rotation of the tube. The tube can be fixed in any desired position by tightening of screw, CS.2.

Part C, Manometer assembly. A, Manometer block; Pl., platform; CS.2, screw for elevation or depression of light beam; pin P which acts as axis for rotation of block A (this axis is parallel to the camera slot); CS.3, screw for rotation of assembly on platform (the axis for this rotation is at right angles to the camera slot).

Part D, Arrangement of manometers.

formed around the membrane. An opening into this chamber allows pressure to be applied to the mirror surface of the membrane, while pressure may be applied to the opposite side through the manometer tube. If the two sides are then connected to the cuff, equal pressures will be applied to both sides of the membrane and no movement will take place. However, a small needle valve is placed in the tube leading to the chamber which enables the rate of pressure change to be controlled so that at any pressure level sudden changes in pressure are not transmitted to the chamber and hence act solely on one side of the membrane. The result is the recording of cuff pulsations at any cuff pressure levels desired.

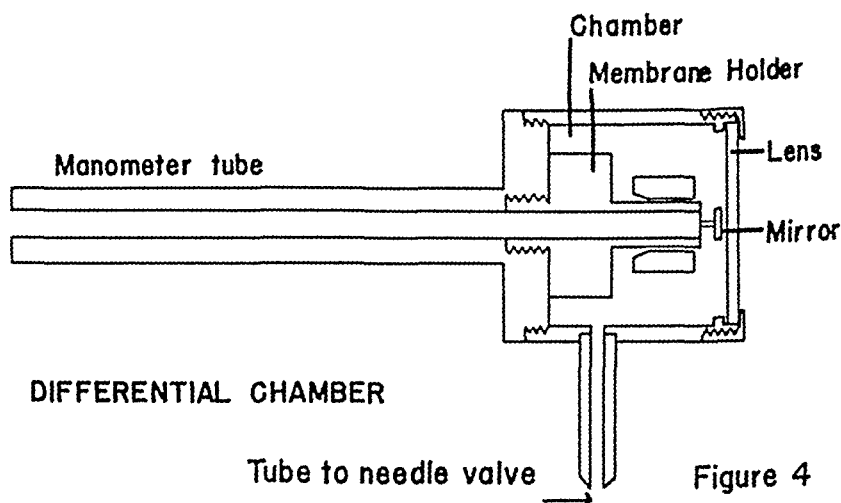


Fig. 4.

*Camera.*—The camera is of the usual type and uses 6-inch seismographic recording paper.

*Records (Fig. 5).*—The types of records obtained by the instrument described are illustrated in Fig. 5, Parts A, B, and C. Fig. 5, Part A, shows the recording of direct arterial pressure (brachial artery) and indirect arterial pressure from the same arm as the cuff pressure is dropped from above to below the systolic level. The time lines are placed across the entire width of this record. In Part B, the time line is placed at the bottom of the record only. This record shows the plethysmographic tracing as well as direct and indirect arterial pressure records and cuff pressure. The recording of venous pressure and respiration is demonstrated in Part C. Here the direct and indirect arterial pressure is recorded as the cuff pressure is decreased from above to below diastolic pressure.

#### SUMMARY

A new instrument is described for simultaneous recording of intraarterial pressure, pneumatic cuff pulsations and pressure, finger plethysmograph, venous pressure, and respiration. It is easily movable and can be used in well-lighted rooms. The principle is similar to that of the Hamilton manometer.

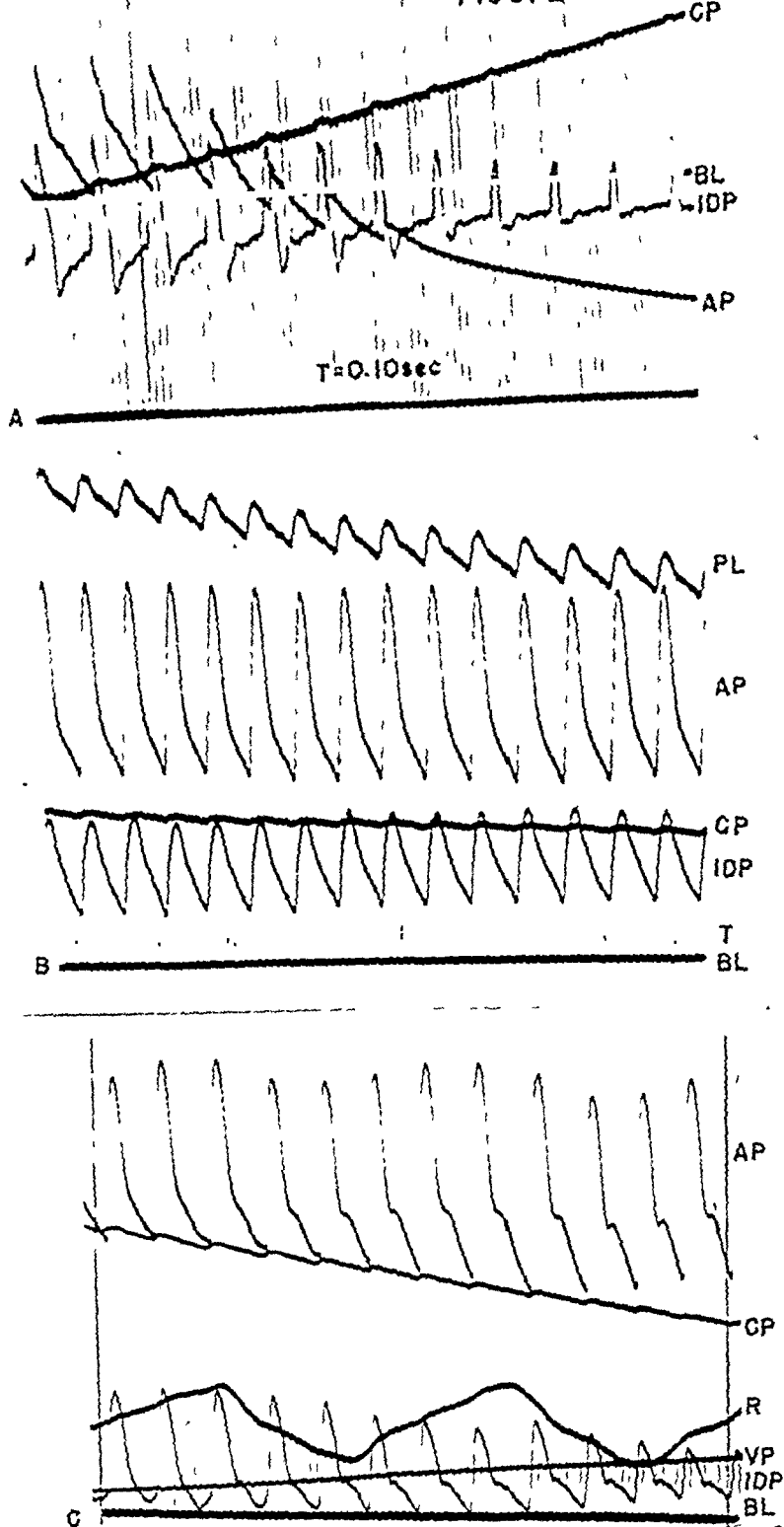


Fig. 5.—Parts A, B, C. Examples of typical records. CP, Cuff pressure; BL, base line; AP, direct arterial pressure; IDP, indirect arterial pressure; PL, plethysmograph; R, respiration; VP, venous pressure.

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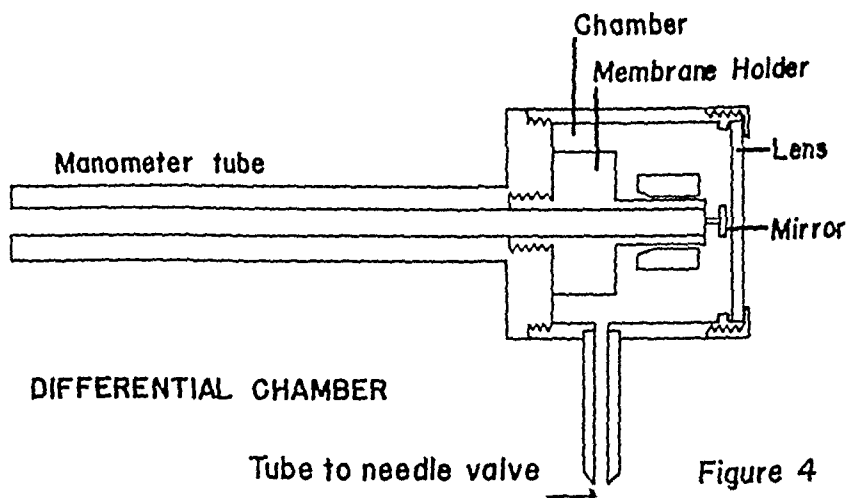


Fig. 4.

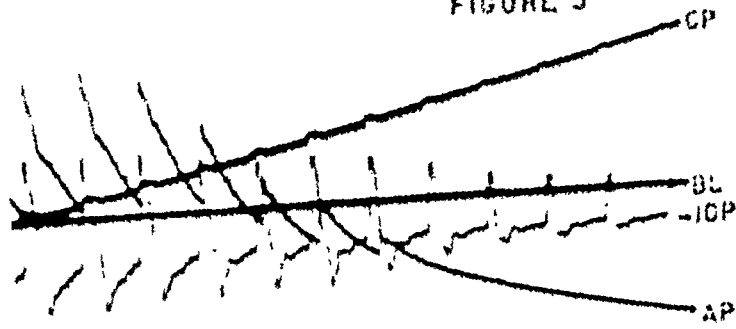
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FIGURE 5



$T=0.10 \text{ sec}$

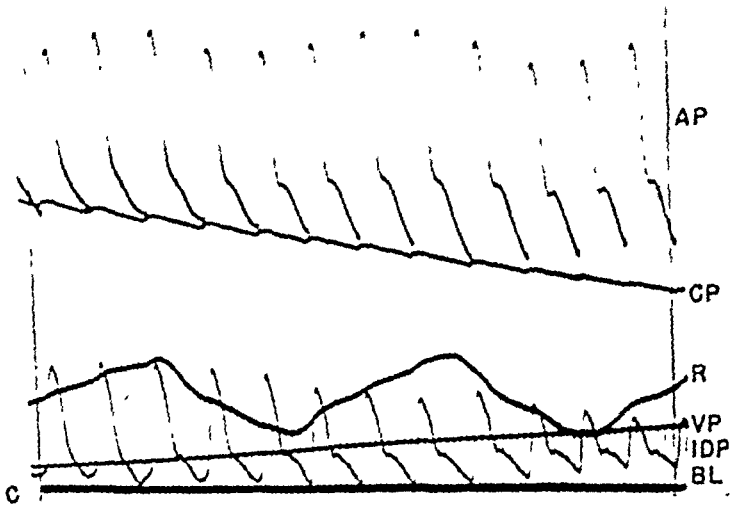
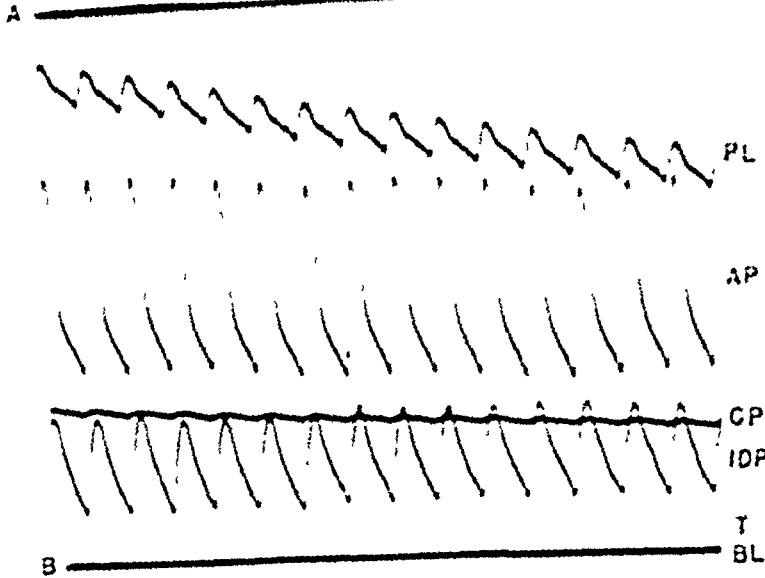


Fig. 5.—Parts A, B, C. Examples of typical records. CP, Cuff pressure. BL, base line; AP, direct arterial pressure; IDP, indirect arterial pressure; PL, plethysmograph; R, respiration; VP, venous pressure.

Plastic tubing is substituted for metal tubes and needles previously used. A differential chamber is described for recording pneumatic cuff pulsations.

## REFERENCES

1. Hamilton, W. F., Brewer, G., and Brotman, I.: Pressure Pulse Contours in Intact Animal; Analytical Description of New High-frequency Hypodermic Manometer With Illustrative Curves of Simultaneous Arterial and Intracardiac Pressures, *Am. J. Physiol.* 107: 427, 1934.
2. Green, H. D.: A Glass Membrane Optical Manometer, *Fed. Proc.* 1: (Part 2): 32, 1942.
3. Gregg, D. E., Eckstein, R. W., and Fineberg, M. H.: Pressure Pulses and Blood Pressure Values in Unanesthetized Dogs, *Am. J. Physiol.* 118: 399, 1937.
4. Kotte, J. H., Iglauer, A., and McGuire, J.: Measurements of Arterial Blood Pressure in Arm and Leg: Comparison of Sphygmomanometric and Direct Intra-arterial Pressures, With Special Attention to Their Relationship in Aortic Regurgitation, *Am. Heart J.* 28: 476, 1944.
5. Cahoon, D. H., Rushmer, R. F., and Kossmann, C. E.: Modification of Hamilton Optical Manometer, *J. LAB. & CLIN. MED.* 30: 541, 1945.

## BOOKS RECEIVED

- Nutritional and Vitamin Therapy in General Practice.** By *Edgar S. Gordon, M.D., Ph.D.*; Associate Professor of Medicine, University of Wisconsin. The Year Book Publishers, Inc., Chicago, 1947. Price \$5.00. With 399 pages and 47 illustrations.
- Functional Cardiovascular Disease.** By *Meyer Friedman, M.D.*; Lieutenant Colonel, Medical Reserve, Corps, U. S. A.; Director, Harold Brunn Institute for Cardiovascular Research, Mt. Zion Hospital; Instructor in Medicine, Stanford Medical School, San Francisco, Calif.; formerly consultant in Medicine and Cardiologist, Tripler General Hospital, Honolulu, T. H. (1942-1945). The Williams & Wilkins Company, Baltimore, 1947. Price \$3.00. With 253 pages.
- Recent Advances in Clinical Pathology.** General Editor *S. C. Dyke, D.M. (Oxon)*, F.R.C.P. (Lond.). Section editors: Bacteriology, *R. Cruickshank, M.D. (Aberd.)*, F.R.C.P. (Lond.); Biochemistry, *E. N. Allott, B.Sc., B.M., B.Ch. (Oxon)*, F.R.C.P. (Lond.); Haematology and Cytology, *B. L. Della Vida, M.D. (Rome)*; Histology, *A. H. T. Robb-Smith, M.D. (Lond.)*. The Blakiston Company, Philadelphia, 1947. With 462 pages, 34 plates, and 19 text figures.
- Rehabilitation Through Better Nutrition—University of Cincinnati Studies in Nutrition at the Hillman Hospital, Birmingham, Alabama.** By *Tom D. Spies, M.D.*; from the Department of Internal Medicine, University of Cincinnati College of Medicine. W. B. Saunders Company, Philadelphia, 1947. Price \$4.00. With 94 pages and 50 figures.
- Penicillin Therapy Including Streptomycin, Tyrothricin and Other Antibiotic Therapy.** By *John A. Kolmer, M.S., M.D., Dr., P.H., Sc.D., LL.D., L.H.D., F.A.C.P.*; Professor of Medicine in the School of Medicine and the School of Dentistry, Temple University; Director of the Research Institute of Cutaneous Medicine; Formerly Professor of Pathology and Bacteriology. Graduate School of Medicine, University of Pennsylvania. D. Appleton-Century Company, Inc., New York, 1947. Price \$6.00. With 323 pages.
- Experiences With Folic Acid.** By *Tom D. Spies, M.D.*; Associate Professor of Medicine, University of Cincinnati School of Medicine; Director of the Nutrition Clinic, Hillman Hospital, Birmingham, Ala. The Year Book Publishers, Inc., Chicago, 1947. Price \$3.75. With 107 pages and 34 illustrations.
- Fundamentals of Clinical Neurology.** By *H. Houston Merritt, M.D.*; Professor of Clinical Neurology, College of Physicians and Surgeons, Columbia University; Chief of Division of Neuropsychiatry, the Montefiore Hospital; *Fred A. Mettler, M.D., Ph.D.*; Associate Professor of Anatomy, College of Physicians and Surgeons, Columbia University; and *Tracy Jackson Putnam, M.D.*; Professor of Neurology and Neurological Surgery, College of Physicians and Surgeons, Columbia University. The Blakiston Company, Philadelphia, 1947. Price \$6.00. With 289 pages and 96 illustrations.
- X-Ray Diffraction Studies in Biology and Medicine.** By *Mona Spiegel-Adolf, M.D.*; Professor of Colloid Chemistry and Head of the Department of Colloid Chemistry, Temple University School of Medicine; and *George C. Henny, M.S., M.D.*; Professor of Medical Physics and Head of the Department of Physics, Temple University School of Medicine. Grune & Stratton, Inc., New York, 1947. Price \$5.50. With 215 pages and 86 figures.
- A Laboratory Manual of Physiological Chemistry.** By *D. Wright Wilson*, Benjamin Rush Professor of Physiological Chemistry, University of Pennsylvania. Sixth edition. The Williams & Wilkins Company, Baltimore, 1947. Price \$2.50. With 275 pages.

- Modern Dermatology and Syphilology.** By *S. William Becker*, M.D.; Clinical Professor of Dermatology, University of Chicago; and *Maximilian E. Obermayer*, M.D.; Clinical Professor and Chairman of the Department of Dermatology, University of Southern California. J. B. Lippincott Company, Philadelphia, 1917. Price \$18.00. Cloth with 1,017 pages, 161 illustrations, and 37 color plates.
- Henrici's Molds, Yeasts, and Actinomycetes.** By *Charles E. Skinner*, Ph.D.; Assistant Professor of Bacteriology, University of Minnesota, Minneapolis, Minn.; *Chester W. Emmons*, Ph.D.; Principal Mycologist, Division of Infectious Diseases, National Institute of Health, Bethesda, Md.; and *Henry M. Tsuchiya*, Ph.D.; Research Associate, Division of Microbiology, Hormel Institute, University of Minnesota, Austin, Minn. John Wiley & Sons, Inc., New York, 1917. Price \$5.00. Cloth with 109 pages and 136 illustrations.
- Practical Physiological Chemistry.** By *Philip B. Hack*, Ph.D.; President, Food Research Laboratories, Inc., Long Island City, N. Y.; *Bernard L. Oser*, Ph.D., Director, Food Research Laboratories, Inc., Long Island City, N. Y.; and *Williams H. Summerson*, Ph.D.; Associate Professor of Biochemistry, Cornell University Medical College, New York City. The Blakiston Company, Philadelphia, 1917. Price \$10.00. Cloth with 1,323 pages, 329 illustrations and 5 color plates.
- Allergy in Theory and Practice.** By *Robert A. Cooke*, M.D., Sc.D., F.A.C.P.; Attending Physician and Director of the Department of Allergy, the Roosevelt Hospital, New York City. W. B. Saunders Company, Philadelphia, 1917. Price \$8.00. Cloth with 372 pages and 43 illustrations.
- Penicillin in Neurology.** By *A. Earl Walker*, M.D.; Associate Professor of Neurological Surgery The University of Chicago; and *Herbert C. Johnson*, M.D.; Resident Neurological Surgeon, The University of Chicago. Charles C. Thomas, Springfield, Ill., 1916. Price \$5.00. Cloth with 179 pages, 26 tables, and 95 illustrations.
- Lehrbuch der Urologie.** By *Prof. Dr. Med. J. Minder*, chem. Ordinarius für Urologie und Direktor der Urolog., Universitäts-Klinik Budapest zur Zeit Spezialarzt der Urologie in Zürich. Hans Huber, Bern, Switzerland 1916. Cloth with 315 pages and 62 figures.
- Outlines of Internal Medicine. Parts I, II, III, IV, V.** Edited by *C. J. Watson*, M.D.; Head, Department of Medicine, University of Minnesota. Fifth edition. William C. Brown Company, Dubuque, Iowa, 1946. Price \$9.00. Paper with 510 pages.
- An Atlas of the Commoner Skin Diseases.** By *Henry C. G. Semon*, M.A., D.M. (Oxon), F.R.C.P. (Lond.); Physician for Diseases of the Skin and Lecturer to Postgraduates, Royal Northern Hospital, Consulting Dermatologist, Hampstead and North West London General Hospital, Ex President and Vice President, Dermatological Section, Royal Society of Medicine, Corresponding Member of the Société Française de Dermatologie et Syphilologie; Médaille de l'Honneur de l'Assistance Publique, République Française; Medical Referee for Industrial Dermatitis, Ministry of National Insurance. Third edition. The Williams & Wilkins Company, Baltimore, 1916. Price \$12.00. Cloth with 341 pages and 139 plates.
- Quantitative Chemical Chemistry. Interpretations. Volume I.** By *John P. Peters*, M.D., M.A.; Professor of Internal Medicine, Yale University School of Medicine; and *Donald D. Van Slyke*, Ph.D., Sc.D.; Member of the Rockefeller Institute for Medical Research. Second edition. The Williams & Wilkins Company, Baltimore, 1946. Price \$7.00. Cloth with 987 pages.



A METHOD OF DETERMINING THE SPECIFIC RENAL FUNCTIONS  
OF GLOMERULAR FILTRATION, MAXIMAL TUBULAR EXCRETION  
(OR REABSORPTION), AND "EFFECTIVE BLOOD FLOW"  
USING A SINGLE INJECTION OF A SINGLE SUBSTANCE

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**M**ETHODS of determining fairly specific renal functions are available at present.<sup>1-3</sup> Because of the complications and detail involved, their usage is limited essentially to the field of research. The following attempt at simplification of these procedures has met with partial success and is presented in the interest of a wider clinical application of these newer and more useful renal function tests. No new concepts are involved, but in order to present our method, the underlying principles will be briefly reviewed from our perspective.

A substance which is excreted solely by being filtered through the glomerular membrane appears in the urine at a rate which is directly proportional to its concentration in the plasma water (Fig. 1). Inulin is an example of this type of compound. The ratio of its urinary excretion per minute to its plasma concentration is constant and may be expressed by the slope of the line indicating inulin excretion at varying plasma concentrations. This represents the inulin clearance, or glomerular filtration rate, in cubic centimeters per minute since it expresses the volume of plasma which contains the amount of inulin excreted in one minute. Inasmuch as the excretion of inulin is accomplished only by glomerular filtration, the slope of this line also represents the glomerular filtration rate; that is, the volume of plasma whose water is filtered by the glomerular membrane in one minute.

A substance which is not only filtered, but which also is excreted by the tubules, will have a higher rate of excretion than inulin. If the tubular excretion is proportional to plasma concentration, then the total excretion will likewise be proportional to plasma concentration, and the ratio  $\frac{\text{urinary excretion rate}}{\text{plasma concentration}}$  will be constant; that is, the clearance will be constant and greater than the inulin clearance. This is generally true of diodrast in low concentrations (Fig. 2).

As their plasma concentration is increased, certain substances can be delivered to the tubules in quantities sufficient to saturate their tubular excretory

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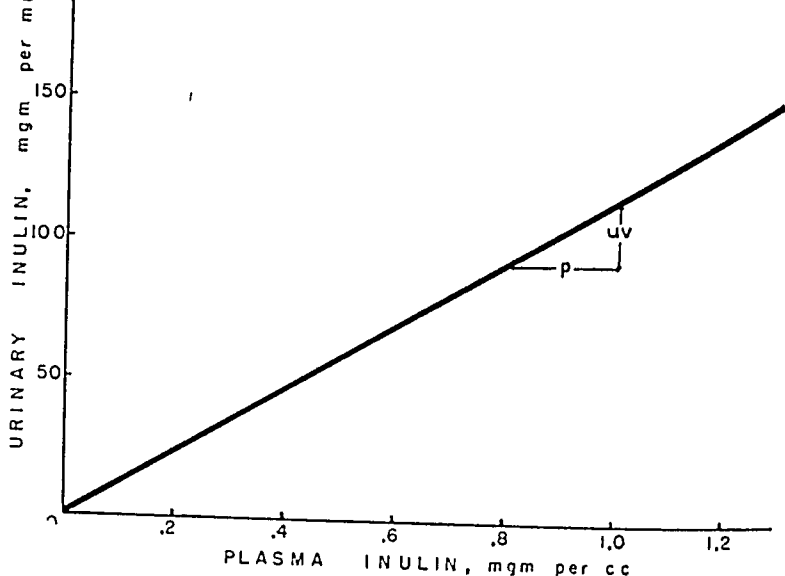


Fig. 1.—The excretion of inulin varies directly with its plasma concentration. The slope of this curve,  $\frac{uv}{p}$ , is constant and represents the plasma clearance of inulin. Since inulin is excreted solely by filtration, the value  $\frac{uv}{p}$  measures the rate of glomerular filtrate formation.

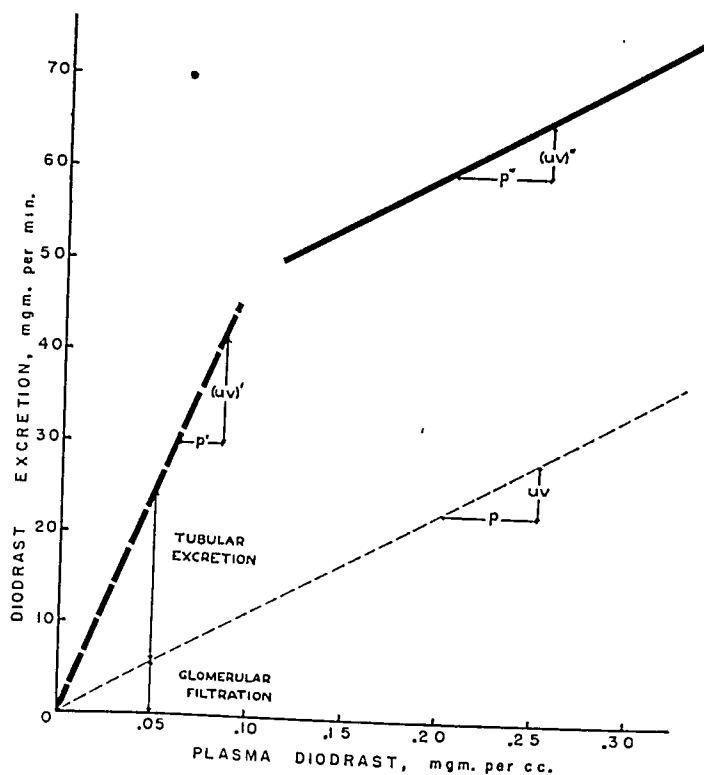


Fig. 2.—For diodrast at low plasma concentrations (heavy dashed line),  $\frac{(uv)'}{p'}$  is constant and represents the diodrast plasma clearance. Since diodrast is excreted by both glomeruli and tubules, the contribution of each may be found if filtration is determined by simultaneous measurement of the inulin, or similar clearance.  
For diodrast at high plasma concentrations, tubular excretion is constant at a maximum ( $T_m$ ), while glomerular excretion continues to increase with increasing plasma concentration. The total excretion in this range (solid line), therefore, parallels the filtration curve, so that  $\frac{\Delta (uv)''}{\Delta p''} = \frac{uv}{p}$  and again measures the glomerular filtration rate.

mechanism. In this case their tubular excretion rate may reach a constant maximum value. The *total* urinary excretion of such substances continues to increase as the plasma level is increased, but only because larger amounts are being filtered. Diodrast, above a plasma total concentration of about 15 mg. per cent, behaves in this fashion and exhibits a constant maximal tubular excretion rate referred to as its  $T_m$  (Fig. 2).

These three functions—glomerular filtration rate, diodrast clearance, and maximal diodrast tubular excretion (or  $T_m$ )—are usually determined by the simultaneous and sustained administration of inulin and diodrast. It may be readily shown theoretically that these functions can be determined by the use of diodrast alone—inulin being unnecessary for the following reason: Since the curve of urinary diodrast excretion in the range of high plasma levels is a constant distance above the curve of diodrast filtration, the two curves are here linear and parallel, and the slope of the curve for total excretion, therefore, again equals the glomerular filtration rate (Fig. 2). Furthermore, the distance the total excretion curve is above the filtration curve represents the maximum tubular excretion or  $T_m$  and can be read on the  $y$  axis by extrapolation. Data for these determinations can be obtained by a single intravenous injection rather than by a continuous intravenous infusion.

#### PLAN OF EXPERIMENTS

Experiments were designed to test the practical validity of this theorem. A single injection of diodrast (or para-amino-hippurate) was given and successive clearance periods obtained as the plasma concentration fell. Inulin was also included in the injection, in order to compare the simultaneous average inulin clearance with the glomerular filtration rate as determined from the curve of diodrast excretion. The following procedure was employed:

Preliminary to, and during, the test a large water intake was provided in order to maintain an adequate, approximately constant urinary flow and extracellular fluid volume. After a blood blank was withdrawn, the substance selected (here diodrast or para-amino-hippurate) was injected intravenously together with inulin in a conveniently small volume of solution. The amounts given were chosen to approximate the desired concentration in a volume of fluid whose mass is equal to 0.25 times body weight. The injection was given conveniently by gravity infusion in five to thirty-five minutes, the rate being controlled to minimize any alterations in blood volume or blood pressure. (Injection of diodrast in less than ten minutes has been noted to produce temporary depression of the diodrast clearance.)

Collection of urine was begun usually twenty-five to forty-five minutes after starting the intravenous injection. Three to twelve (average, seven) urinary samples were obtained, usually successively, each representing periods of nine to forty-six (usually ten to twenty-five) minutes. The usual technique for collection<sup>2</sup> was followed, using a large indwelling catheter and three successive quick rinsings of the bladder with 20 c.c. of saline, timing the end of rinsing with an accuracy within five to fifteen seconds.

Three or more blood samples were drawn at intervals to establish curves of plasma concentration. Duplicate or triplicate analyses of the heparinized blood plasma and of the diluted urine samples were made in the usual manner.<sup>1-3</sup> The plasma concentrations corresponding to two and one-half minutes before the mid-point of each collection period were read from the curve of log plasma concentration plotted against time. In the case of diodrast or para-amino-hippurate, these values were corrected for the water content of plasma and for protein binding, being expressed as milligrams of unbound material per cubic centimeter of plasma water. The average urinary excretion rates corresponding to each of these values were determined by dividing the total excretion of material in milligrams for each period by the length of the period in minutes. The excretion rates were then plotted as ordinates against the plasma concentrations as abscissas.

To all data in the region of maximal tubular function, a straight line was applied by the method of least squares. (This also can be done fairly accurately by inspection.) The selection of points can usually be made if it is remembered that the curve turns toward the origin at low values. The slope of the line obtained represents the average glomerular filtration rate in cubic centimeters per minute, and the extrapolated intercept on the y axis indicates the  $T_m$  in milligrams per minute. While this is termed a "graphic" method, the values also can be obtained readily from the formula for the line derived by the method of least squares. (The  $T_m$  is the value for y where  $x = 0$ , and the glomerular filtration rate is  $\frac{dy}{dx}$ .)

If urinary collections are continued, data may be obtained for low plasma levels. Here the slope of the curve increases to approach a straight line running through the origin. The slope of this line represents the "effective renal plasma flow."

## RESULTS

Comparisons have been made in fourteen subjects in the following manner: (1) The glomerular filtration rate (GFR), derived by the aforementioned "graphic" method from diodrast or para-amino-hippurate excretions, was compared with the average of the simultaneous inulin clearance determinations ( $C_I$ ) for each subject. (2) From the conventional calculations based on simultaneous excretions of diodrast (or PAH) and inulin,  $T_m$ 's were obtained, and the average of these  $T_m$ 's for each subject was compared with the "graphic" determination of  $T_m$  from diodrast (or PAH) excretion values alone. (3) When sufficiently low plasma levels were reached, the graphic and calculated "effective renal blood flow" were compared. (4) In several cases the modified procedure for the "graphic" method followed or preceded the "standard" method of maintaining continuous infusion at approximately constant plasma level, and the consecutive results were compared.

Representative curves are shown in Fig. 3, illustrating variation in grouping of points, self-depression of  $T_m$  due to too rapid injection, and clearances at high and low plasma levels of diodrast and PAH. The italicized values are

those calculated from the simultaneous inulin determinations. The comparisons are summarized in Table I. Of special significance are columns 5 and 6 and 10 and 11. Columns 5 and 6 indicate an average difference between methods of determining filtration rate of 8 c.c. per minute or 13 per cent, regardless of sign.

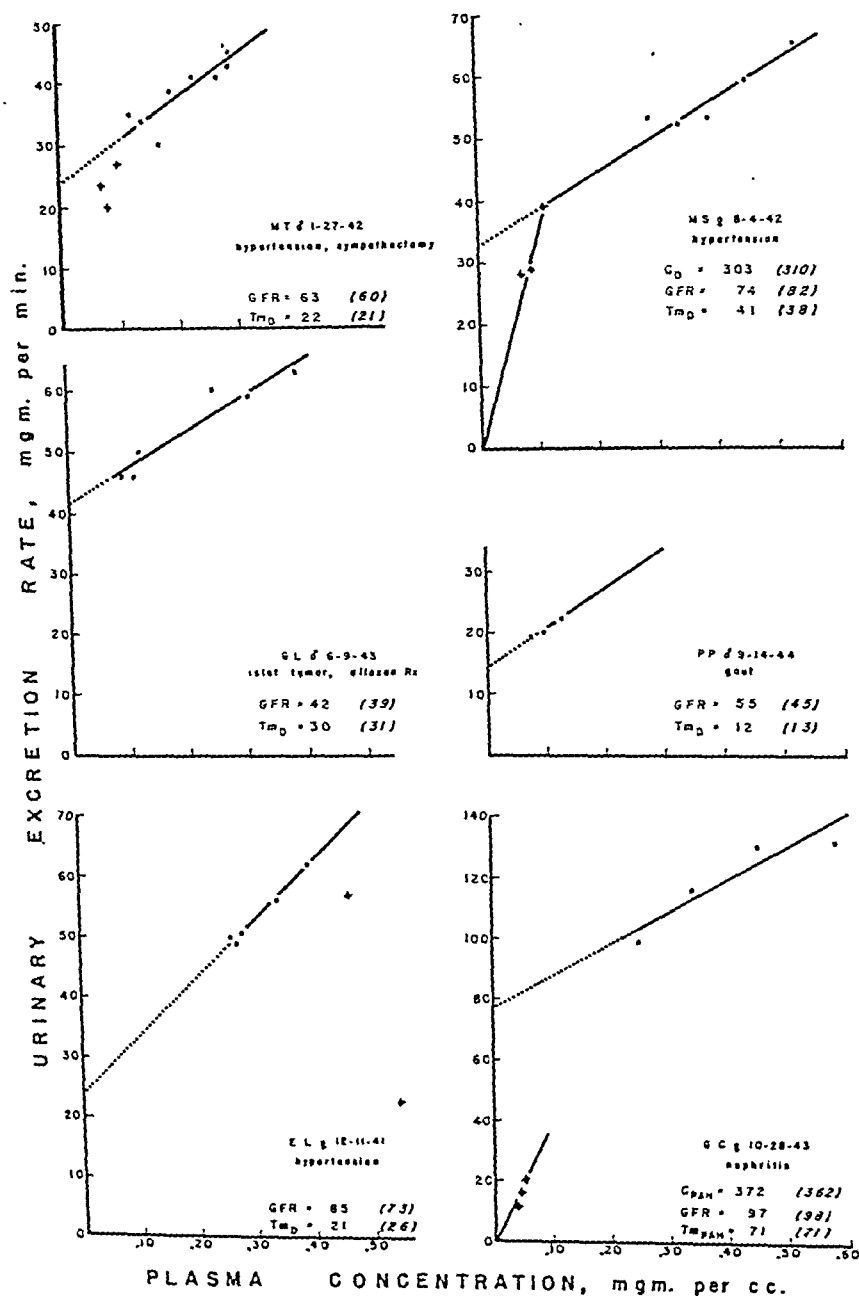


Fig. 3.—Representative experiments showing determination of renal functions by single injection technique. Simultaneous values as determined by standard technique are indicated in italics. (Data indicated as - do not apply to determination of glomerular filtration rate, (GFR) or  $Tm$ . Discussed in text.)

TABLE I. SUMMARY OF COMPARISONS OF SIMPLIFIED METHOD WITH STANDARD METHOD OF DETERMINING GLOMERULAR FILTRATION AND T<sub>m</sub>  
 (DISCUSSED IN TEXT)

1	2	3	4	5	6	7	8	9	10	11
SUBJECT	GLOMERULAR FILTRATION RATE (SIMPLIFIED METHOD)	AVERAGE INULIN CLEAR- ANCE	AVERAGE DEVIATION OF INULIN CLEAR- ANCES	DIFFERENCE BETWEEN METHODS (COLUMNS 2-3)		T <sub>m</sub> (SIMPLI- FIED METHOD)	AVERAGE T <sub>m</sub> (STANDARD METHOD)	AVERAGE DEVIATION OF T <sub>m</sub>	DIFFERENCE BETWEEN METHODS (COLUMNS 7-8)	
	C.C./MIN.	C.C./MIN.	PER CENT	C.C./MIN.	PER CENT	MG./MIN.	MG./MIN.	PER CENT	MG./MIN.	PER CENT
J.M.	81	97	6	-16	-16	35	27	7	8	30
D.M.	54	64	4	-10	-16	30	26	4	4	15
L.W.	89	86	5	3	3	35	37	5	-2	-5
E.L.	85	73	5	12	16	21	26	7	-5	-19
M.T.	63	60	9	3	5	22	21	9	1	5
J.D.	44	33	9	11	32	13	16	6	-3	-19
R.N.	56	63	16	-7	-11	23	19	13	4	21
J.S.	52	63	5	-11	-21	32	30	5	2	7
J.P.	89	80	3	9	11	44	46	2	-2	-4
C.M.	70	66	3	4	6	28	29	4	-1	-3
M.S.	74	82	5	-8	-10	41	39	5	2	5
G.L.	42	39	10	3	8	30	31	4	-1	-3
G.C.	97	98	3	-1	-1	71*	71*	4	0	0
P.P.	55	45	12	10	22	12	13	6	-1	-8
Average	68	68	7	8†	13†	31	31	6	3†	10†

\*Para-amino-hippurate. All others are diodrast iodine.

†Regardless of sign.

This is to be contrasted with the average of the average deviations for the several inulin clearances in each experiment (column 4), indicating an average variation of 7 per cent in the determinations by the standard method itself. For the Tm comparisons, the average difference between the standard and the simplified method was 3 mg. per minute or 10 per cent, neglecting sign (columns 10 and 11), while by contrast the average of the average deviations of the standard Tm calculations was 6 per cent (column 9). The identity of the averages of the fourteen values for the two methods, both for filtration rates and for Tm, is, in view of the small number of cases, largely a prejudicing coincidence and does not argue for the reliability of the modified method in the individual case.

#### DISCUSSION

*Errors.*—This modification shares with all methods of measuring renal excretory function the errors arising from possible misunderstanding of the mode of excretion of the substance being studied, particularly in conditions of disease. Sampling errors are slightly greater than in the standard techniques, because of shorter rinsing, but are probably well within the analytical errors common to both methods.

There are, in addition, three errors introduced by the use of a changing plasma concentration rather than a sustained one. The first is that a representative sample of the mean plasma concentration may not be obtained. If the linear or the logarithmic rate of change of plasma concentration should happen to be constant, then the mean concentration can be read on a suitable graphic plot from the mid-point in time, or calculated from the values at the beginning ( $P_1$ ) and end ( $P_2$ ) of the period. Where the plasma concentration fall is linear, the mean concentration is equal to  $\frac{P_1 + P_2}{2}$ . Where the logarithm of concen-

tration falls in a linear manner (as is the case with inulin and approximately so with diodrast at high plasma levels) the mean concentration is

$$\frac{P_1 - P_2}{2.302 \log \frac{P_1}{P_2}}.$$

For any relationship of plasma concentration and time, the true mean concentration can be arrived at by determining the entire area under the concentration curve for the period, either analytically or planimetrically, and dividing by the length of the period. In forty-two periods a comparison was made of the values as calculated by these three methods: from mid-point in time, by logarithmic plot, and by planimetric measurement. The differences between the values so derived (Table II) did not fall in any consistent direction and were less than the errors inherent in the remainder of the procedure; therefore, the simple "mid-point" value was taken as representative of the mean plasma concentration.

The second error is due to the fact that urine reaching the bladder is derived from plasma a short while previously, and, therefore, does not correspond to the simultaneous plasma concentration. This anachronism is due to

TABLE II. DIFFERENCES IN "MEAN PLASMA CONCENTRATION" AS OBTAINED BY (A) ARITHMETIC MEAN (B) LOGARITHMIC MEAN (C) PLANIMETRIC MEAN VALUES, ALL EXPRESSED AS PER CENT OF VALUE

COMPARISON	AVERAGE DIFFERENCE	MAXIMUM	MINIMUM
A-B	0.5	0.9	-0.8
A-C	0.1	0.6	-0.9
B-C	0.7	1.5	-0.5

a delay in the passage of urine through the kidney and ureters to the bladder, which is relatively greater than the circulatory delay in the passage of blood from the kidney to the arm vein (if venous blood is used). This time lag is variable and was found by Smith and co-workers<sup>1</sup> to average about two and one-half minutes in man. If the plasma concentration is constant no error results, but if the plasma level is rising or falling some correction should be considered. Applying a correction of two and one-half minutes to a curve of falling concentrations produces higher values for mean concentration, and therefore lower clearances, than if no correction were made. Using the conventional method of calculating clearances, but with a falling plasma concentration curve, the average decrease in clearance in thirty-one determinations was 2 per cent (the maximum, 4.3 per cent; the minimum, 0.8 per cent) when a two and one-half minute correction was applied. Conventionally calculating Tm from inulin and diodrast excretions based on a falling curve, a two and one-half minute correction resulted in even less change, since the Tm is derived by clearance differences. The average correction was -0.8 per cent; the range was from -3.0 per cent to +1.6 per cent. We have therefore applied the correction, concluding that the estimated anachronism of two and one-half minutes can be in error by 100 per cent without introducing significant error in result, except perhaps when very rapid changes in plasma level are occurring.

The third possible source of error arises in case of storage of material by the kidney, to be released during falling plasma concentration. Smith and co-workers<sup>1</sup> have concluded that there is no appreciable storage of inulin or of diodrast since clearances were the same whether obtained when plasma concentrations were rising or falling.

*Technique of Test.*—An important technical difficulty is presented in obtaining the proper range of blood levels. If renal function is high, or periods too long, too few points may fall within the usable range. On the other hand, if periods are too brief, inaccuracy in urine collection increases; or if renal function is too poor, the change in plasma level may not yield a sufficient scatter of points, unless the test be prolonged unduly. It is therefore imperative that some prior estimate of function be made in each case, as for instance by urea clearance, phenolsulfonphthalein, or concentration tests. When determinations of effective renal blood flow are also desired, it may take some time until sufficiently low plasma concentrations are reached in the case of large subjects, or in subjects with low renal function. In such cases the test may be interrupted and resumed if intermediate points are not desired.



*Usefulness of Method.*—In determining “effective renal plasma flow,” this method offers an advantage over the standard one in that a check is provided on the constancy of renal extraction at different plasma concentrations. In renal disease the demonstration of a linear relation between excretion and plasma concentration of diodrast (or PAH) may be necessary before the clearance can be considered to represent “effective renal plasma flow.”<sup>4</sup>

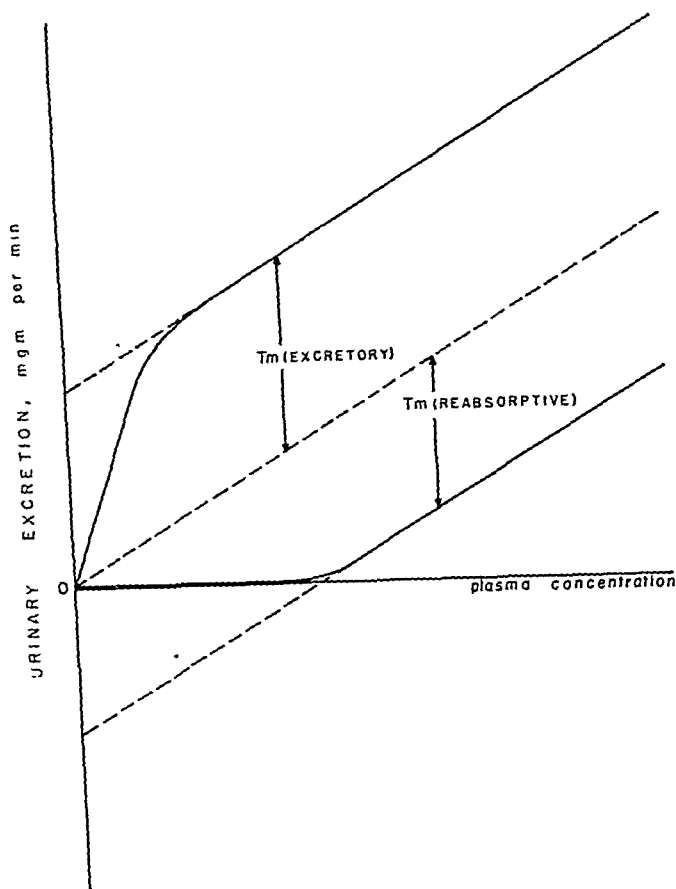


Fig. 4.—Various substances exhibiting a maximal tubular transport ( $T_m$ ), either excretory or reabsorptive, may be used to determine the glomerular filtration rate as well.

By plotting excretion rates against the *total* concentration in the water of the plasma rather than against the concentration of *free* diodrast (or PAH), the  $T_m$  value will be unchanged, but the value for glomerular filtration rate will be low by a factor equal to the average per cent of material bound by protein. Smith and associates<sup>3</sup> have, in fact, adopted this method to enable them to check the degree of protein binding of PAH.

It is inaccurate to rely upon this method to estimate filtration fractions or to derive other hemodynamic ratios, for not only are the determinations of “effective blood flow” and glomerular filtration not made simultaneously, but the lessened accuracy of the determinations may yield disproportionate inaccuracy in any calculation involving ratios of the values obtained.

The fact that the calculations and analytic detail have been simplified should not suggest that the procedure has achieved true simplicity. We have not had a sufficient experience to predict whether it can be developed to a point where all the pitfalls of the more accurate clearance studies can be consistently avoided.

The method is applicable not only to diodrast, but also to any substance which exhibits a maximal tubular transport, either excretory or reabsorptive (Fig. 4). Data from the literature on hippuran, glucose, and ascorbic acid, when replotted by our method, finds agreement with the original calculations (Table III). Because the analysis of para-amino-hippurate is within the range of many clinical chemical laboratories, this substance is particularly suited to the method. The ideal substance is, however, still to be found, for it must possess the attributes of being nontoxic, not metabolized, not stored, nor rapidly excreted via other routes; must not appreciably or variably be bound to protein, must be readily subject to analysis, and its normal  $T_m$  should be large in relation to the error of analysis. With a substance possessing these attributes, a widespread clinical application of this simplified procedure may be feasible.

TABLE III. COMPARISON OF SIMPLIFIED METHOD WITH STANDARD METHOD OF DETERMINING GLOMERULAR FILTRATION AND  $T_m$   
(Data from the literature; graph drawn by inspection)

SUB- STANCE	SUBJECT	SIMPLIFIED METHOD		STANDARD METHOD			REFERENCE
		GLOMER- ULAR FILTRA- TION RATE	$T_m$	CREA- TININE CLEAR- ANCE	INULIN CLEAR- ANCE	$T_m$	
		C.C./MIN.	MG./MIN.	C.C./MIN.	C.C./MIN.	MG./MIN.	
Hippuran	Dog (3 experiments)	65	7.5	65.7		7.6	5 (Table III)
Diodrast	Dog	43	13.2	45		11.9	5 (Table II)
Glucose	Dog	117	292	115.2		268	6 (Table I)
Glucose	Dog	80	215	81.5		220	7 (Table I)
Ascorbic acid	Man (6 experiments)	130	2.5		124.2	2.17	8 (Table I)

*Other Simplified Methods.*—Other workers have reported on simplifications in the determination of these renal functions. The method of Findley and co-workers,<sup>9</sup> which assumes that the per cent of injected diodrast excreted in the urine in thirty minutes is a measure of  $T_m$ , has no physiologic basis. Applying his method to data determined by standard procedure has yielded errors of several hundred per cent.

Foà and Foà have performed clearances during falling plasma concentrations.<sup>10</sup> Our studies establish the validity of such procedure and offer further simplifications.

Earle and Berliner recently have suggested<sup>11</sup> that a sustaining infusion can be given to maintain a constant blood level after equilibrium has been established, when the rate of infusion can be taken to indicate excretion in place of the urinary collections, thus obviating the need of catheterization. How one

establishes the proper level of infusion other than by approximation is not indicated. It is not feasible to perform "spot" blood analyses during the test, and slow drifts in plasma concentration of insignificant degree may be produced by infusions at rates which are considerably different from the rate of urinary loss. For example, an infusion of 20 per cent more inulin than is being excreted will take twenty minutes to increase the plasma concentration by 2 per cent under conditions where plasma level is 100 mg. per cent, the total inulin in the body is 15 Gm., and the rate of excretion is 75 mg. per minute. If extracellular fluid volume remains constant over periods long enough to provide accuracy, then the procedure may have great practicality.

Newman and associates<sup>12</sup> have proposed that the rate of change in plasma level of mannitol after equilibrium has been established can be taken to yield the rate of excretion. We had endeavored to establish this possibility for inulin and diodrast. Disappointingly, we found that the theoretic volume of solution (obtained by dividing the amount of material remaining in the body by its plasma level) continued to rise and did not attain equilibrium during the several hour course of the experiment. Mannitol appears to equilibrate sooner than inulin and/or high concentrations of diodrast, but data establishing the correctness of the assumed equilibration do not appear in the literature. If the volume of distribution could be as reliably known, or held constant, in the case of substances in plasma concentration high enough to exhibit a  $T_m$ , then the mathematic derivatives of  $T_m$  without urinary collection may become possible. In this method of approach also, a small percentage change in volume of distribution accounts for an amount of test material which is large in proportion to the rate of excretion, especially at high plasma levels.

#### SUMMARY

Certain specific renal functions may be determined with sufficient accuracy for clinical purposes by blood and urine analyses after a single intravenous injection of one of several substances.

The theoretic implications and the present practical limitations of the method are discussed.

#### REFERENCES

1. Smith, H. W., Goldring, W., and Chasis, H.: The Measurement of the Tubular Excretory Mass, Effective Blood Flow and Filtration Rate in the Normal Human Kidney, *J. Clin. Investigation* 17: 263, 1938.
2. Alving, A. S., and Miller, B. F.: A Practical Method for the Measurement of Glomerular Filtration Rate (Inulin Clearance). *Arch. Int. Med.* 66: 306, 1940.
3. Smith, H. W., Finkelstein, N., Alimosa, L., Crawford, B., and Graber, M.: The Renal Clearances of Substituted Hippuric Acid Derivatives and Other Aromatic Acids in Dog and Man, *J. Clin. Investigation* 24: 388, 1945.
4. Earle, D. P., Jr., Taggart, J. V., and Shannon, J. A.: Glomerulonephritis; Survey of Functional Organization of Kidney in Various Stages of Diffuse Glomerulonephritis, *J. Clin. Investigation* 23: 119, 1944.
5. Elsom, K. A., Bott, P. A., and Shiels, E. H.: On the Excretion of Skiodan, Diodrast and Hippuran by the Dog, *Am. J. Physiol.* 115: 548, 1936.
6. Shannon, J. A., and Fisher, S.: The Renal Tubular Resorption of Glucose in the Normal Dog, *Am. J. Physiol.* 122: 765, 1938.
7. Shannon, J. A., Farber, S., and Troast, L.: The Measurement of Glucose  $T_m$  in the Normal Dog, *Am. J. Physiol.* 133: 752, 1941.
8. Ralli, E. P., Friedman, G. J., and Rubin, S. H.: The Mechanism of the Excretion of Vitamin C by the Human Kidney, *J. Clin. Investigation* 17: 763, 1938.

9. Findley, T., Edwards, J. C., Clinton, E., and White, H. L.: A Simplified Technique for Measuring Renal Blood Flow and Tubular Excretory Mass, *J. LAB. & CLIN. MED.* 28: 916, 1943.
10. Foà, P. P., and Foà, N. L.: A Simple Method for Determining Effective Renal Blood Flow and Tubular Excretory Mass in Man, *Proc. Soc. Exper. Biol. & Med.* 51: 375, 1942.
11. Earle, D. P., Jr., and Berliner, R. W.: A Simplified Clinical Procedure for Measurement of Glomerular Filtration Rate and Renal Plasma Flow, *Proc. Soc. Exper. Biol. & Med.* 62: 262, 1946.
12. Newman, E. V., Bordley, J., III, and Winternitz, J.: The Interrelationships of Glomerular Filtration Rate (Mannitol Clearance), Extracellular Fluid Volume, Surface Area of the Body, and Plasma Concentration of Mannitol, *Bull. Johns Hopkins Hosp.* 75: 253, 1944.

## RADIOACTIVE PHOSPHORUS, $P^{32}$ : A SIX-YEAR CLINICAL EVALUATION OF INTERNAL RADIATION THERAPY

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FOR nearly half a century roentgen radiation has been the most effective palliative treatment for the more chronic leucemic states and allied hematologic dyscrasias, having been first used by Senn in a case of lymphatic leucemia in 1903.<sup>1</sup> There are, however, distinct and obvious limitations in the clinical application of roentgen-ray therapy as well as of radium and other naturally occurring radioactive elements. These have been utilized more or less effectively in medicine by way of external application, but these elements have proved dangerous when deposited within the body as internal therapy. With the development of the cyclotron by Ernest Lawrence, both physicist and biologist were provided with a tool by means of which a wide range of important hitherto stable chemical elements in nature might be rendered temporarily unstable and radioactive for a limited period of time, thus opening up entirely new fields of scientific exploration and control.

In medicine, the diversion of small quantities of selected isotopes essential to normal body metabolism has made possible important tracer element studies, as well as selective internal radiation therapy. Very recently the provision for utilizing the government-owned uranium piles as a source of some of these biologically significant radioactive isotopes is permitting the more rapid development of this field of knowledge through a much greater potential supply of the desired elements.

Of the artificially produced isotopes, radioactive phosphorus ( $P^{32}$ ) has been studied most extensively to date as a means of internal radiation therapy. It became evident following the earliest observations in animals that this isotope might be used advantageously in certain human hematologic dyscrasias. Clinical reports evaluating radioactive phosphorus as a therapeutic agent have been made by Low-Beer, Lawrence, and Stone,<sup>2</sup> Erf, Tuttle, and Lawrence,<sup>4</sup> Fitz-Hugh and Hodes,<sup>5</sup> Kenney,<sup>6</sup> Craver,<sup>7</sup> Warren,<sup>8</sup> Hall, Watkins, Hargraves, and Giffin,<sup>9</sup> Erf,<sup>10, 11</sup> and Hoster and Doan,<sup>12</sup> Reinhard, Moore, Bierbaum, and Moore<sup>13</sup> have recently published a comprehensive and detailed summary of the

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basic concepts and accumulated clinical experience in this field as a background for the critical analysis of their own observations which renders any repetition of these details at this time unnecessary and inappropriate.

Studies in this laboratory and clinic were initiated in 1941\* with radioactive phosphorus from the Radiation Laboratory at Berkeley and were continued with material from the Ohio State University cyclotron when it became functional in 1942. We are now regularly receiving radioactive phosphorus and other isotopes through a government contract from the Manhattan Project, Oak Ridge, Tenn. An analysis of the results to date in our first 100 patients treated with radioactive phosphorus  $P^{32}$  is presented in this communication (Table I).

TABLE I. DISEASES TREATED WITH RADIOACTIVE PHOSPHORUS

Polycythemia rubra vera	20
The leucemias	
Lymphatic	
Acute (leucosarcoma)	18
Chronic	11
Myelogenous	
Acute	1
Chronic	13
Monocytic	
Acute	9
Chronic	1
Hodgkin's syndrome	16
Metastatic carcinoma and sarcoma	5
Multiple myeloma	4
Mycosis fungoides	1
Exfoliative dermatitis	1
Total	100

#### MATERIALS AND METHODS

The base line laboratory studies on all patients included serial complete total white, red blood cell, and platelet counts, white cell differentials using the supravital technique, hemoglobin determinations, reticulocyte percentages, sedimentation rate, and packed cell volume. Repeated bone marrow examinations via sternal aspiration and the supravital technique were made. Determinations of the basal metabolic rate were done routinely, excluding any thyroid dysfunction by the iodine fractionation techniques of Curtis and associates.<sup>14</sup> Appropriate biopsies were performed as the basis for fixed section diagnoses in the carcinomatoses, Hodgkin's syndromes, and mycosis fungoides. Blood volume determinations, using Evans blue dye T-1824,<sup>15</sup> formed a part of the study in patients with polycythemia rubra vera. Complete hematologic studies were made daily in those patients being observed in the University Hospital. Those who were seen as out patients had the same determinations made at intervals of one to eight weeks, depending upon the acuteness or chronicity of the clinical course and the availability of the patient. Certified United States Bureau of Standards Trenner blood cell pipettes were used. The sedimentation rate was estimated by the Wintrobe method as modified for cell volume by Rourke and Ernestine.<sup>16</sup> A modification of the Dameshek method was used for platelet and reticulocyte determinations. Hemoglobin measurements were made by the oxyhemoglobin determinations on the Evelyn photoelectric colorimeter, which was standardized by the Van Slyke apparatus.

*Radioactive Phosphorus.*—The details of the cyclotron bombardment of the red phosphorus have been adequately considered elsewhere.<sup>17</sup> The phosphorus isotope was converted

\*Through the courtesy of Dr. John Lawrence.

into dibasic sodium phosphate by the method described by Kamen.<sup>13</sup> The route of administration in the earlier studies was determined by the availability and purity of the material. To conserve P<sup>32</sup> the intravenous route was used most frequently, this being more economical by approximately 30 per cent. The more recent radioactive phosphorus from the uranium piles

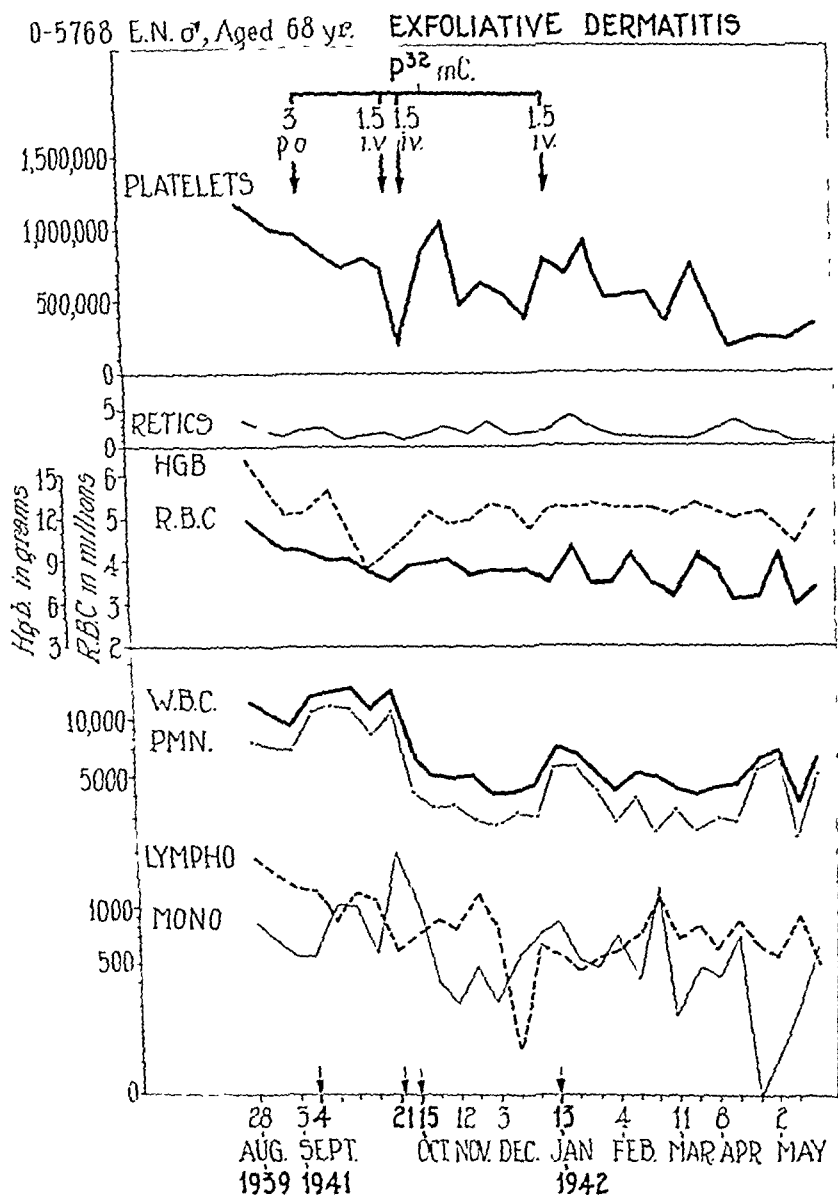


Fig. 1.—E. N., 68-year-old white man. Diagnosis: Exfoliative dermatitis of undetermined origin. Normal bone marrow and peripheral blood.

Under P<sup>32</sup> therapy there was improvement in the skin lesions, with corresponding disappearance of the pruritus. Note the progressive depression and decline in the platelet, erythrocyte, hemoglobin, granulocyte, lymphocyte, and monocyte levels during and after the course of P<sup>32</sup>. These hematologic changes fortunately re-equilibrated at subclinical levels but serve to warn against the too-free administration of radioactive isotopes where normal hematopoiesis may be primarily concerned.

at Oak Ridge has been supplied as potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ), and, due to the relatively large amount of potassium, administration has been confined to the oral route.

The Geiger counter has been used in making the measurements of radioactivity.

Phosphorus is selectively deposited in bone and in the nuclei of rapidly multiplying cells. The radioactive and nonradioactive isotopes of phosphorus have identical chemical properties. Thus it is possible for the beta rays emitted by the radioactive phosphorus deposited at these sites to exert maximum inhibitory and destructive effects on adjacent marrow elements. The effect on normal marrow and peripheral blood is illustrated in Fig. 1, data on a patient with resistant exfoliative dermatitis who responded to small doses of radioactive phosphorus with definite but subclinical hematologic side effects.

### POLYCYTHEMIA RUBRA VERA

In polycythemia rubra vera there is a characteristic *triune hyperplasia* of all marrow elements—myeloid, erythroid, and megakaryocytic. With the therapeutic means that have been previously employed in this syndrome, including venesection, phenylhydrazine, and various erythrottoxins, adequate control of the hyperplasia of all of the marrow elements has been unsatisfactory.

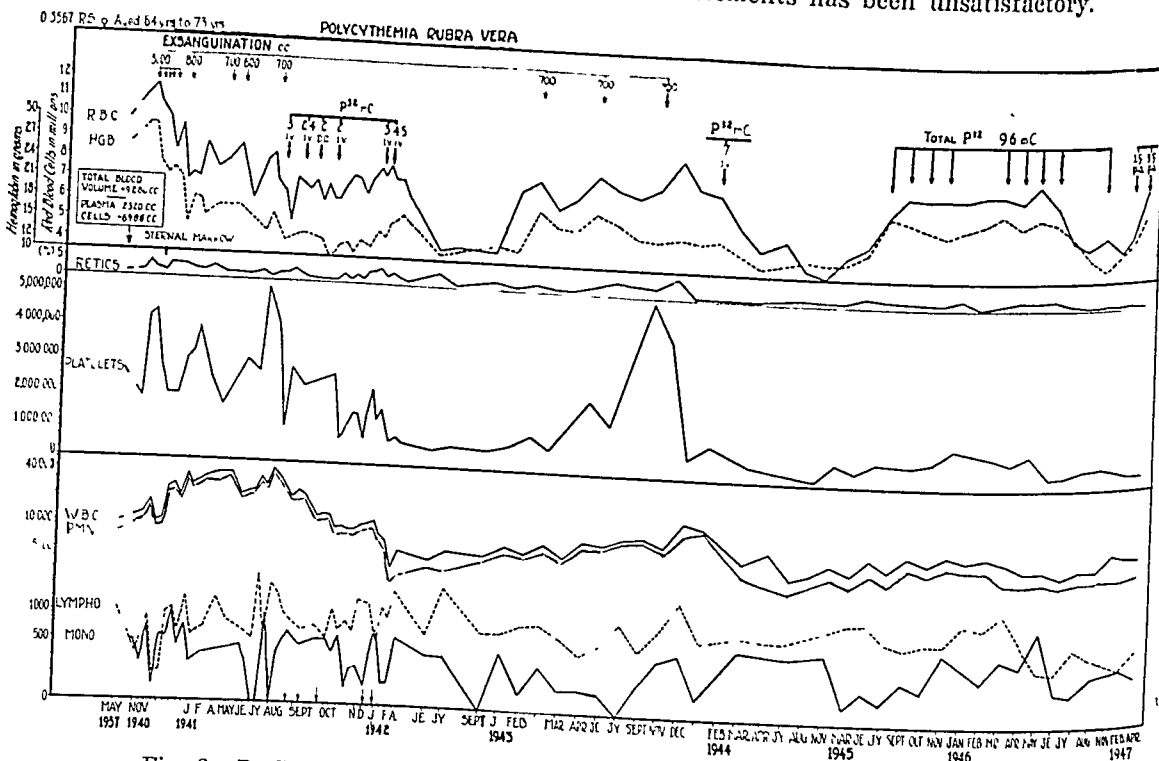


Fig. 2.—R. S., 73-year-old white woman. Diagnosis: Polycythemia rubra vera.

Venesection during the first four years of observation, while reducing the erythrocyte level, failed completely to control either the white blood cell or the platelet levels which actually rose steadily with accompanying clinical symptoms. The leucemoid picture was promptly to 1941 and again in 1943, and the critical response to  $\text{P}^{32}$  on each occasion. Since 1941, three complete hematologic and clinical remissions have been induced with  $\text{P}^{32}$  therapy lasting respectively twelve months, seventeen months, and, currently, seven months. An excellent example of the control of the *triune marrow hyperplasia* with internal radiation therapy.



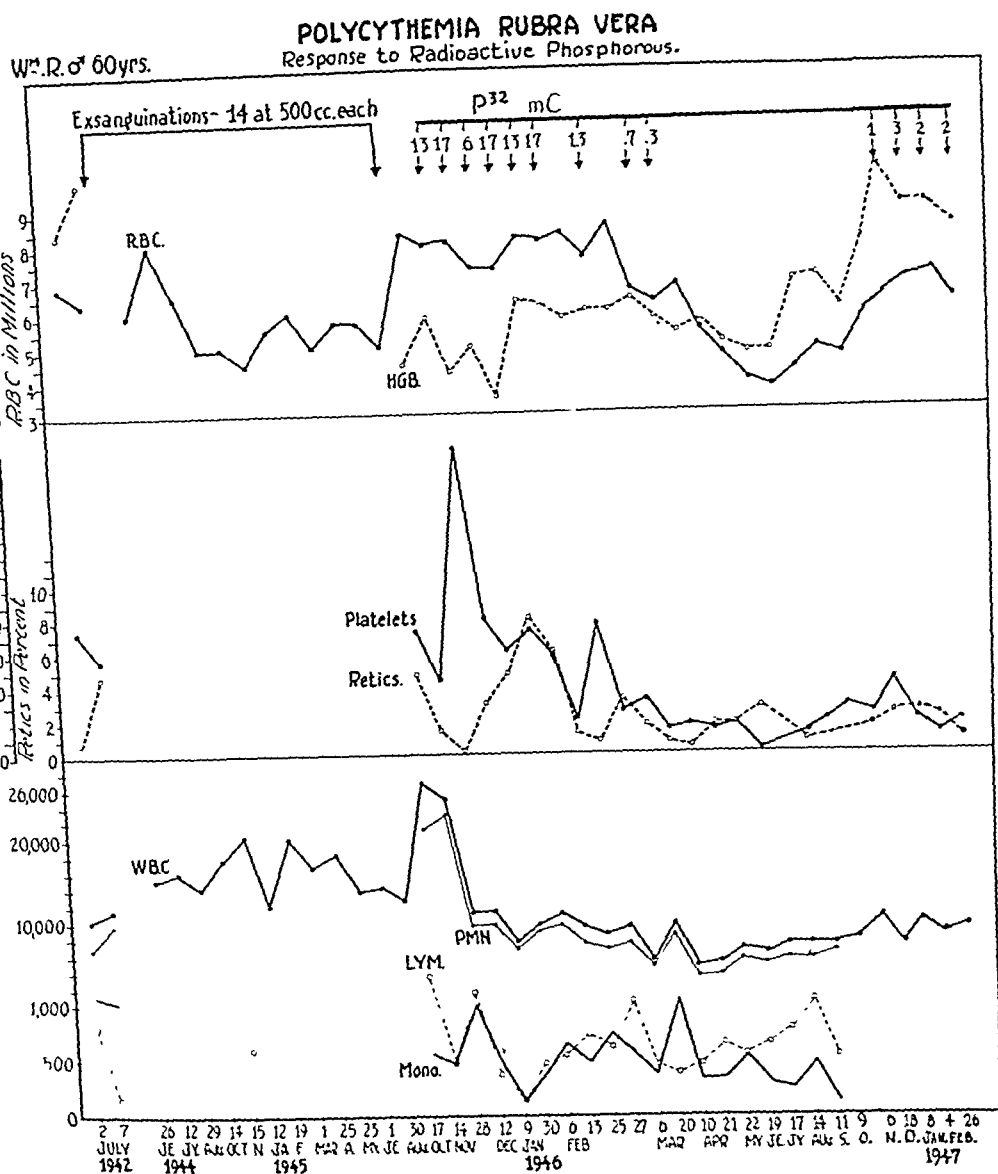


Fig. 3.—W. R., 60-year-old white man. Diagnosis: Polycythemia rubra vera.

During the three months preceding the establishment of the diagnosis in 1942 this patient had both cerebral and coronary thromboses. Venesections and roentgen radiation together failed to maintain a satisfactory hematologic or clinical equilibrium during the next three years. A total of 10.6 mc. P<sup>32</sup> administered in small doses over a four-month period induced a remission of one year. Second remission now being induced. Complete relief of the incapacitating vertigo and recurrent intravascular thromboses has been effected. Note the response of the platelets (8,000,000 per cubic millimeter) and granulocytes (26,000 per cubic millimeter) as well as erythrocytes (8,300,000 per cubic millimeter) to this therapy.



A total of twenty patients with this diagnosis, twelve men and eight women, have been treated in this series (Table II). The ages range from 28 to 73 years. Only two have expired during this period of observation, one from injuries received in an automobile accident and the other from carcinoma of the bladder. Excellent hematologic and clinical remissions lasting from five to nineteen months were effected in ten (Figs. 2 to 4). Seven patients showed partial responses. Four of these required venesection at various times in association with the  $P^{32}$  therapy. Two (Cases 7 and 11) had a fall in both white blood cells and platelets to a low normal before the red blood cells had shown a satisfactory response. In both of these instances venesection seemed indicated to bring the red blood cell level to normal since it was feared that further internal radiation therapy might cause a clinically significant granulocytopenia or thrombocytopenia. Thrombocytopenia, neutropenia, and anemia have been noted as complications of  $P^{32}$  therapy,<sup>18</sup> and the necessity for careful individualization of dosage cannot be overemphasized. Another case (Case 12) has been seen only at infrequent intervals over the past three years. Only when pruritus became severe was medical attention sought.  $P^{32}$  controlled the pruritus satisfactorily. In the limited dosage then available, however, little effect was observed on the hematologic picture. During the earlier years of this study the supply of radioactive phosphorus was very limited, and in the majority of instances where satisfactory remissions were not induced the  $P^{32}$  dosage was definitely inadequate. No patient with this diagnosis has developed a true irreversible leucemic, hematologic, or clinical picture in our experience, though a "leucemoid" blood picture has not been unusual.

#### LYMPHATIC LEUCEMIA

Ten of the eleven patients with chronic lymphatic leucemia (Table III) treated with  $P^{32}$  have expired. One is still under therapy (Fig. 5). Excellent clinical and hematologic improvement was obtained in three patients, one of whom (Fig. 6) had developed a marked hypersensitivity to roentgen radiation. Only moderate improvement was effected in three cases and slight improvement in two. In three patients no change in either clinical or hematologic status was apparent; two of these previously had become resistant to roentgen radiation. Three patients with intractable pruritus associated with leucemia cutis were relieved with  $P^{32}$  therapy, one of these having received no relief previously from spray roentgen radiation.

#### LEUCOSARCOMA

##### (LYMPHOSARCOMA CELL LEUCEMIA)

Fifteen of the eighteen patients with leucosarcoma treated in this series were children under 9 years of age (Table IV). In twelve cases temporary remissions, both clinical and hematologic, were induced, lasting from three days to three weeks; however, the leucemic process then became more resistant to radiation therapy and occasionally appeared actually to be accentuated (Fig. 7). All of these patients have expired.

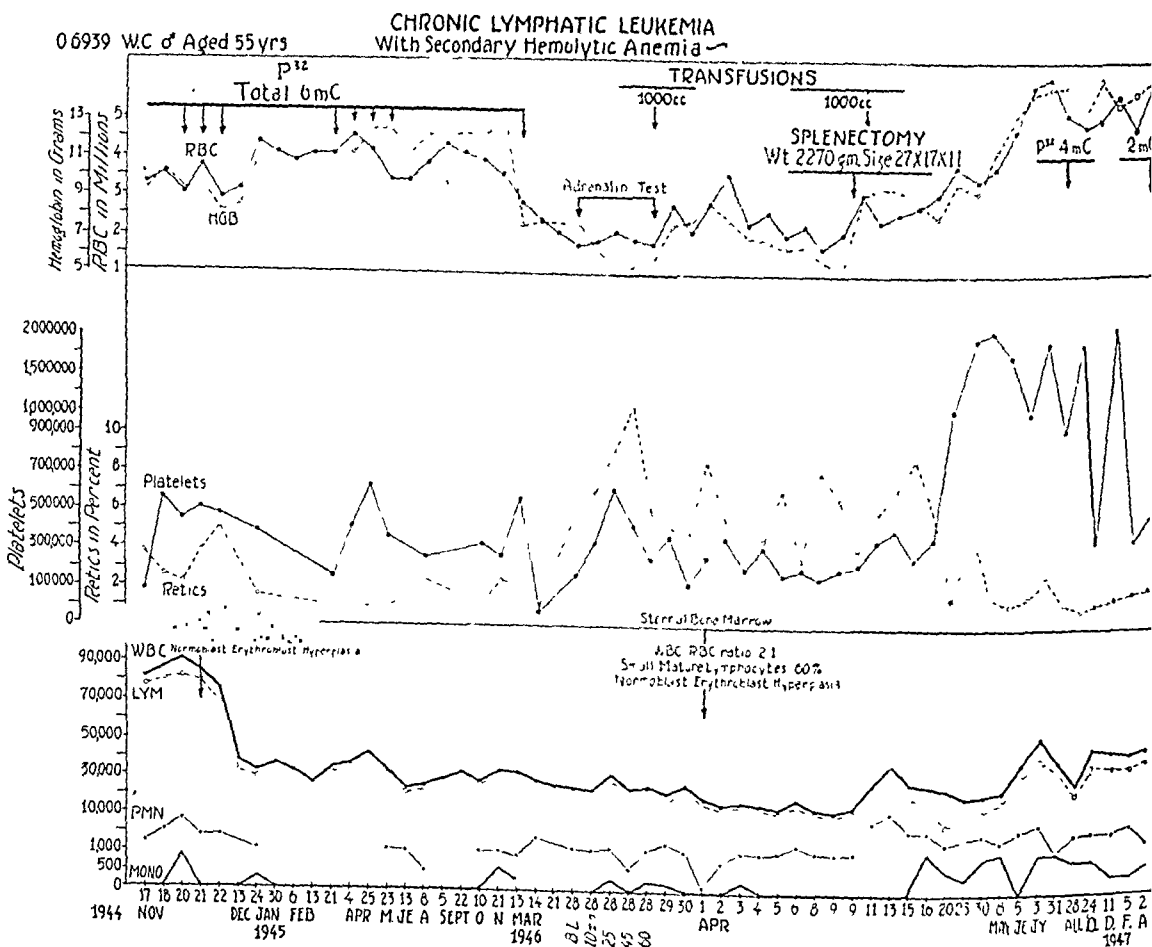


Fig. 5.—W. C., 55-year-old white man. Diagnosis: Chronic lymphatic leucemia.

Radioactive phosphorus reduced the white blood cells from 90,000 to 20,000 with a reciprocal rise in the circulating red blood cells and hemoglobin levels; the patient was asymptomatic for a sixteen-month period from November, 1944, to March, 1946, on this regime. Then there suddenly developed a subacute erythroclastic crisis with clinical jaundice and a rapid fall in red cells and hemoglobin during a three-week period. The reticulocytes became moderately elevated (6 to 11 per cent). The spleen increased rapidly in size but there was no corresponding enlargement of any of the regional lymph nodes, and the peripheral white cell and lymphocyte equilibrium showed no significant qualitative or quantitative alterations. The bone marrow revealed a W.B.C.:R.B.C. ratio of 2:1 with 60 per cent small mature lymphocytes present, but there was striking evidence of normoblastic and erythroblastic overcompensatory hyperplasia. There was no sign of excessive destruction of marrow elements by the radioactive phosphorus. The adrenalin test further supported a diagnosis of hypersplenism, and splenectomy was advised. Following splenectomy clinical and hematologic equilibrium was promptly re-established. This is an unquestionable example of secondary hypersplenism developing during the course of chronic lymphatic leucemia but unassociated with any generalized constitutional leucemic exacerbation. The remission continues with complete clinical recovery and return to full occupation as of June 1, 1947.

## MYELOGENOUS LEUCEMIA

Fourteen patients with myelogenous leucemia have been treated with radioactive phosphorus (Table V). All were chronically ill from a clinical and hematologic point of view except one, a patient with an acute basophile granulocyte leucemia. The criteria for dosage included not only the maintenance of a satisfactory total white blood count, but also of adequate erythrocyte and hemoglobin levels. These latter objectives were satisfactorily accomplished in most instances.

Control of the splenomegaly in these patients proved to be much more difficult than the lowering of the peripheral white cell count (Fig. 8). Frequently roentgen radiation over the spleen had to be invoked to relieve the symptoms referable to the mechanical discomfort occasioned by the massive enlargement of this organ. More intensive P<sup>32</sup> therapy was contraindicated because of peripheral blood and bone marrow findings.

In this limited series of cases of myelogenous leucemia, radioactive phosphorus has not proved to be as effective a therapeutic agent on the whole as roentgen radiation (Fig. 9). It is, however, a very useful therapeutic agent in those

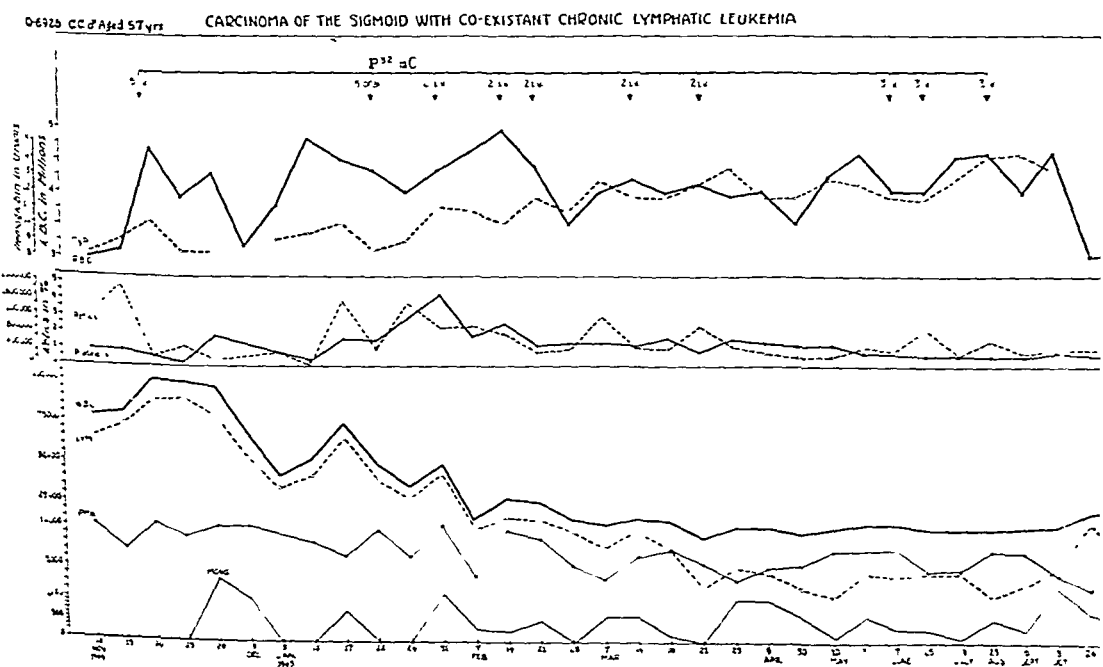


Fig. 6.—C. C., 57-year-old white man. Diagnosis: 1. Chronic lymphatic leucemia and 2. carcinoma of the lower bowel.

Medical attention was sought first by this patient for a lower bowel obstruction. Carcinoma of the sigmoid was diagnosed on biopsy. Coexistent lymphatic leucemia was found.

The leucemic process was brought under excellent control with radioactive phosphorus therapy, the white blood count, red cell count, and platelets being maintained near normal values. The carcinomatosis progressed and was the cause of death. At the time of death the leucemic process was under excellent control.

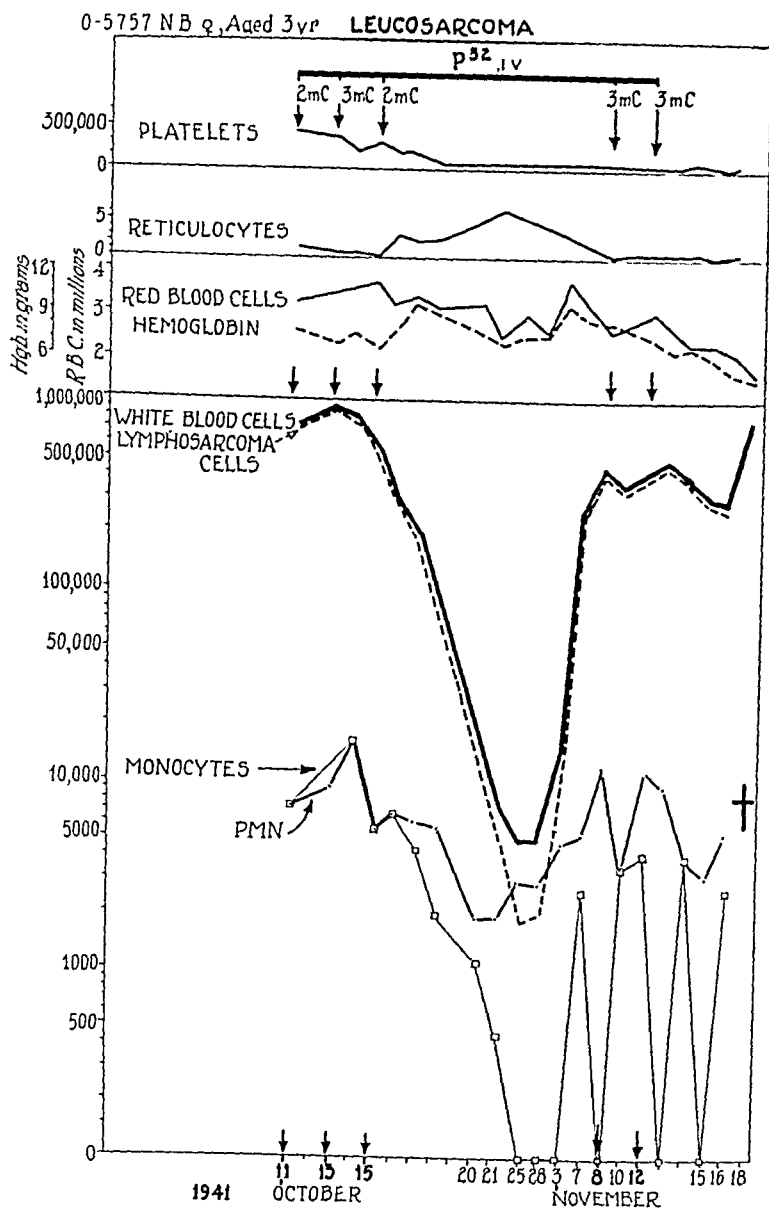


Fig. 7.—N. B., 3-year-old white girl. Diagnosis: Leucosarcoma.

With 7 mc. of radioactive phosphorus over a four-day period there was a precipitous drop in the white blood count from a high of 966,000 per cubic millimeter to 5,000, with a marked regression of the generalized lymphadenopathy and splenomegaly. This clinical and hematologic remission lasted only three weeks. The second course of  $P^{32}$  therapy failed to have any clinical or hematologic effect.

patients who develop an intolerance to roentgen radiation, with intractable and incapacitating constitutional reactions to even very small dosages, as was the case in three patients in this series.

### MONOCYTIC LEUCEMIA

Eight of the nine patients with monocytic leukemia treated in this series were clinically acutely ill (Table VI). In the one chronically ill patient, mod-

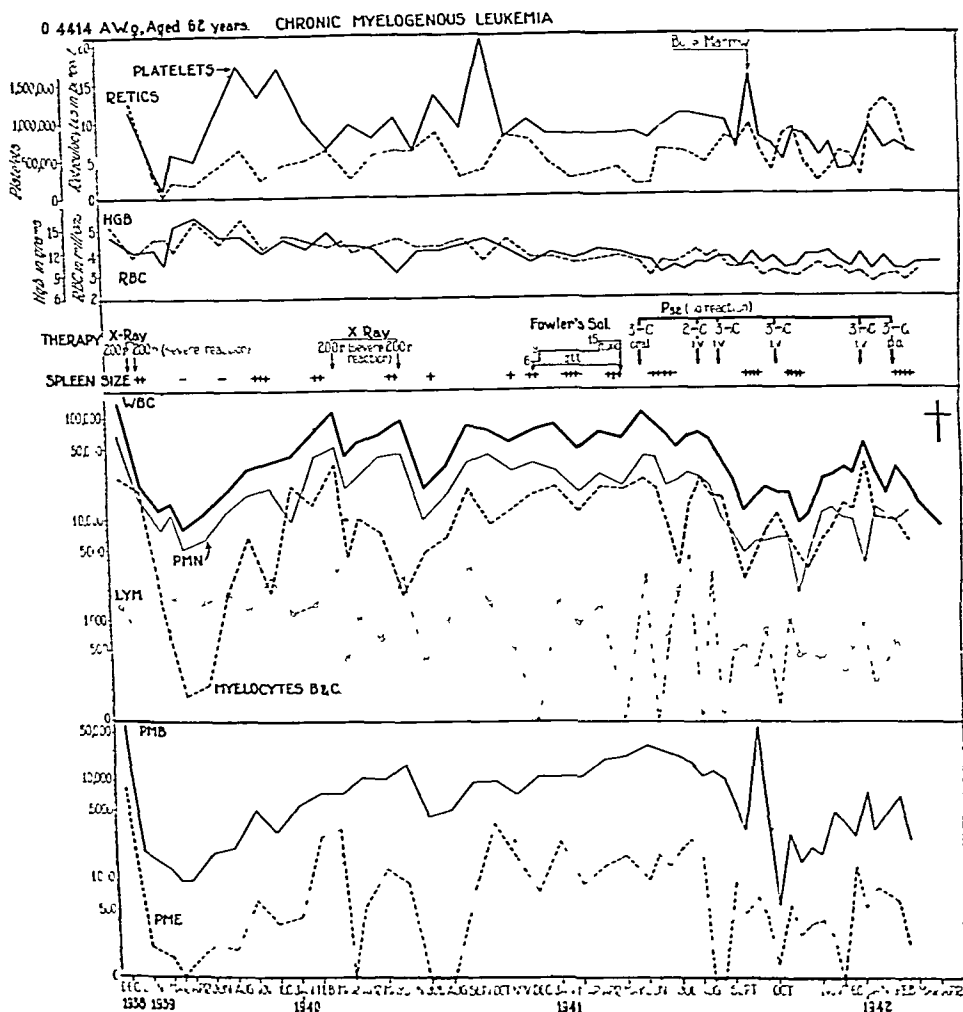


Fig. 8.—A. W., 63-year-old white woman. Diagnosis: Chronic myelogenous leukemia.

The myelogenous leucemia in this patient responded initially to roentgen radiation with prompt reduction in the splenomegaly; however, she experienced severe radiation sickness following each treatment. A six-month trial of Fowler's solution failed to accomplish satisfactory results within the tolerance of the patient for arsenic.  $P^{32}$  was then instituted without any chemical reactions. The peripheral hematologic picture was quickly and adequately controlled. The splenomegaly, which was the source of most of the subjective complaints, continued to progress and became chiefly responsible for the terminal intestinal obstruction.

erate clinical improvement followed the use of  $P^{32}$  over a twenty-one-month period. In only three instances was a temporary favorable effect induced in those acutely ill, lasting from two to four weeks (Fig. 10).

### HODGKIN'S DISEASE

Eleven of the sixteen patients with Hodgkin's syndrome treated with radioactive phosphorus in this clinic have been reported previously by Hoster and Doan.<sup>12</sup> In none of these was significant benefit from this form of therapy noted. There were several instances in which a dangerous depression in thrombocytes, erythrocytes, and leucocytes occurred incidental to the  $P^{32}$  therapy.

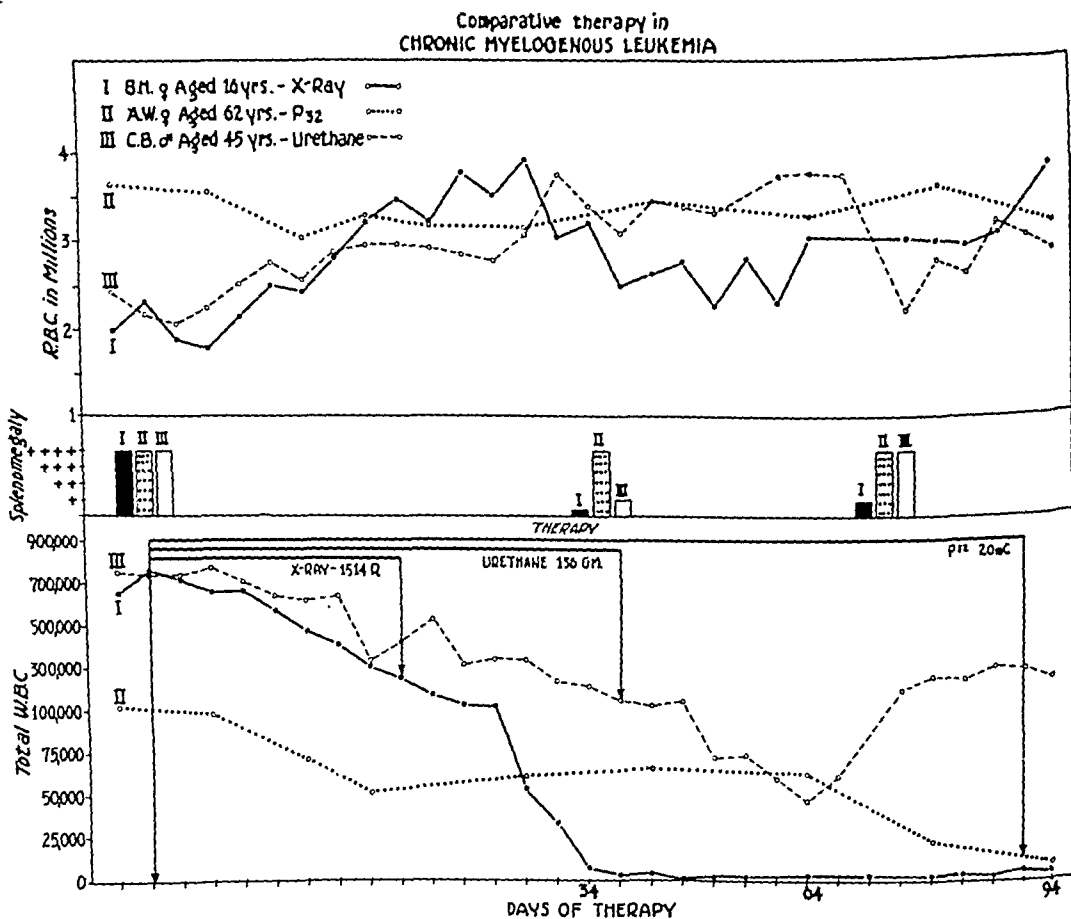


Fig. 9.—Comparative effectiveness of (I) external roentgen irradiation, (II) internal radiation with  $P^{32}$ , and (III) oral urethane chemotherapy in three patients with chronic myelogenous leucemia. Both spleen size and excessive circulating white blood cells were controlled most effectively by roentgen irradiation; the remission in this patient is continuing after six months.  $P^{32}$  reduced the elevated white count but had no effect on the splenomegaly. Urethane produced a transient clinical remission with a fall in the total circulating white cells and rapid decrease in size of the spleen. Upon discontinuing the daily urethane dosage there was an immediate rise in white count with concurrent re-enlargement of the spleen.



## MISCELLANEOUS

A small group of patients (four with multiple myeloma, five having malignancies with metastases to bone, one with exfoliative dermatitis of undetermined etiology, and one with mycosis fungoides, Table VII) may be evaluated together. In the patients with malignancies involving bone, temporary relief from pain was obtained, lasting from two weeks to six months; however, no appreciable regression in the lesions could be recognized roentgenologically or clinically.

The cutaneous lesions, associated with a marked pruritus in one patient with exfoliative dermatitis, were significantly improved under  $P^{32}$  therapy; however, the bone marrow, which had been normal at the time therapy was started in this patient, reflected the depressant effect of the radioactivity and provided

J.W. Aged 32 yrs. ♂

## ACUTE MONOCYTIC LEUKEMIA

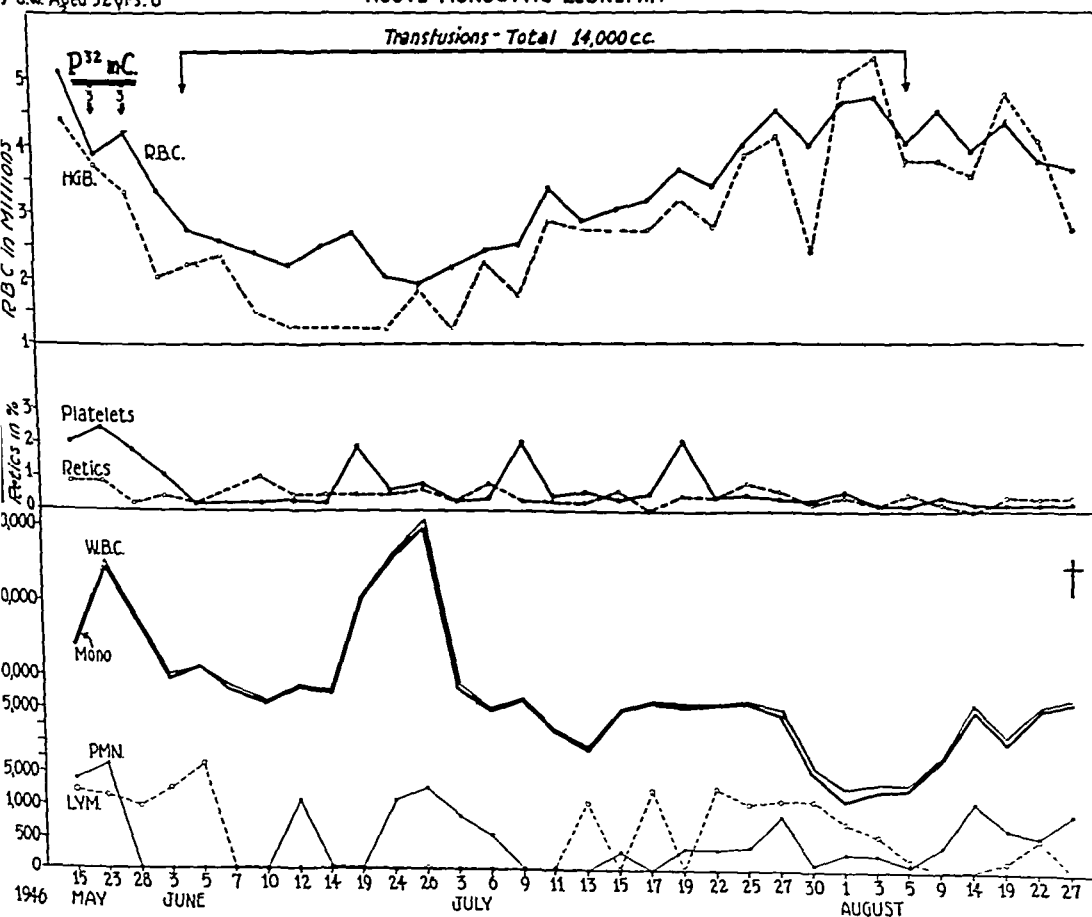


Fig. 10.—Following 6 mc. of  $P^{32}$  there was a fall in the white blood count from 253,000 to 88,000, during which regression it was still necessary to support the patient with repeated blood transfusions, and neither circulating reticulocytes nor platelets nor granulocytes reflected any regression in marrow invasion by monoblasts. The efficacy of any specific therapy under such circumstances is, of course, very questionable.

the chief criterion upon which the dosage tolerance was established. The patient with mycosis fungoides was under observation for two months. The course was steadily downhill, and he expired without receiving any apparent benefit from therapy.

Aside from the temporary relief of bone pain and pruritus, it is felt that the phosphorus radioactive isotope therapy had no significant effect on the course of the disease in any of these patients.

### CONCLUSIONS

1. Radioactive phosphorus has been, in this clinic to date, of greatest value in controlling the clinical and hematologic manifestations of polycythemia rubra vera.

2.  $P^{32}$  is a valuable adjunct to other therapy in certain of the more chronic leucemic states, particularly in those patients intolerant of or resistant to roentgen radiation. The splenomegaly in chronic myeloid leucemia has frequently proved more resistant to  $P^{32}$  than to deep x-ray therapy.

3. Although internal radiation therapy may cause an occasional brief favorable effect in the acute leucemias, all too frequently  $P^{32}$  has appeared to accentuate the clinical acuteness of the leucemic process and has never proved permanently beneficial in our hands.

4. Radioactive phosphorus has failed effectively to control Hodgkin's syndrome in our clinic and may threaten the integrity of the marrow unless great care and discretion are observed in the dosages used.

5. The deep bone pain which characterizes metastatic malignancies to bone may be relieved by  $P^{32}$ . The evidence as to specific retardation of the lesions remains questionable.

6. Pruritus secondary to leucemia cutis, polycythemia rubra vera, and exfoliative dermatitis has been effectively controlled by radioactive phosphorus therapy in selected instances.

7. Our source of radioactive phosphorus until recently has been the University of California (Berkeley) and Ohio State University cyclotrons. The supply of isotopes has been correspondingly limited. The recent decision to release radioactive phosphorus, as well as other radioactive isotopes, from the uranium piles of the Manhattan Project for medical and biologic use will make this form of internal radiation much more readily available to clinical investigators. *It must be emphasized that the efficacy of this therapy depends upon "selective" cell destruction. There is a wide variance in individual susceptibility and tissue response. Extreme care in administering internal radiation therapy is therefore essential. Dosage must be carefully adjusted to each patient's needs and to his idiosyncrasies as determined individually. Our experience suggests small, well-spaced doses of radioactive phosphorus with frequent, careful, complete blood and bone marrow studies to insure the integrity of normal hematopoiesis during this treatment of human diseases.*

TABLE II. RADIOACTIVE PHOSPHORUS THERAPY IN POLYCYTHEMIA RUBRA VERA

CASE	PATIENT	AGE, RACE, AND SEX	ONSET OF THERAPY				P <sup>32</sup> DOSAGE* (MG.)	MINIMAL COUNTS UNDER P <sup>32</sup> THERAPY				IM-PROVEMENT OF SYMPTOMS	REMARKS
			R.B.C. (PER C.MM.)	HEMO-GLO-BIN (GM. %)	W.B.C. (PER C.MM.)	PLATE-LETS (PER C.MM.)		R.B.C. (PER C.MM.)	HEMO-GLO-BIN (GM. %)	W.B.C. (PER C.MM.)	PLATE-LETS (PER C.MM.)		
1	T. G.	64, W., M.	6.7	13.1	35,000	2,492,600	7.5 over 5 mo.	5.0	10.1	18,000	991,800	+++	Good remission for one year; treatment discontinued due to limited amount of P <sup>32</sup>
2	L. J. S.	58, W., M.	(1) 6.9	12.6	37,000	2,603,104	16 over 9 mo. (o)	5.8	10.4	5,450	384,400	+	Initial therapy only moderately satisfactory, then a 14-month period of venesections; second course was effecting satisfactory control when patient was killed in automobile accident, 30 days after last therapy
3	E. W. S.	47, W., M.	(2) 7.0	14.6	17,650	229,240	7 (o)	6.0	10.1			++	Five-month follow up with good control clinically and hematologically; note W.B.C. and platelets particularly; no further studies
4	H. S.	66, W., F.	6.5	16.9	16,150	3,308,760	3 (o)	7.3	13.4	10,150	2,769,780	?	One small oral dose P <sup>32</sup> only; insufficient therapy available
5	H. D.	55, W., M.	7.8	18.0	17,900	789,000	3 (o)					?	One small dose P <sup>32</sup> ; uncooperative
6	E. B.	40, W., M.	8.0	15.9	19,000	2,880,000	5.6 over 3 mo.	3.9	12.9	6,450	249,000	+++	Remission 8 months after initial course; has maintained satisfactory hemogram within normal values, with additional therapy as indicated
7	J. V.	28, W., M.	7.74	20.1	5,750	913,320	3.6 over 8 mo. 5.7 over 8 mo.	4.4	12.1	3,150	378,540	+++	Phenylhydrazine therapy for 9 months preceding P <sup>32</sup> ; Neutropenia developed; R.B.C. elevated; 2 venesections (totaling 1,250 c.c.) made; remission 3 months
8	Z. R.	54, W., F.	7.2	12.9	6,300	1,388,100	2.8 over 4 mo.	4.2	11.0	3,550	339,000	+++	Four months necessary to induce remission which has been satisfactorily held for 14 months and continues
9	B. F.	58, W., M.	6.7	12.8	11,450	349,440	7.0 over 7 mo.	5.54	10.9	9,450	232,680	++	Venesections for 2 years preceding P <sup>32</sup> therapy; venesections totaling 1,500 c.c. required along with the P <sup>32</sup> to maintain red cell and hemoglobin equilibrium

\*All therapy intravenous unless otherwise indicated.

TABLE II—Cont'd

CASE	PATIENT	AGE, RACE, AND SEX	ONSET OF THERAPY					MINIMAL COUNTS UNDER P <sub>32</sub> THERAPY				IM- PROVE- MENT OF SYMP- TOMS	REMARKS
			R.B.C. (PER C.M.M.)	H.E.MO- GLO- BIN (GM. %)	W.B.C. (PER C.M.M.)	PLATE- LETS (PER C.M.M.)	P <sub>32</sub> DOSAGE* (MC.)	R.B.C. (PER C.M.M.)	H.E.MO- GLO- BIN (GM. %)	W.B.C. (PER C.M.M.)	PLATE- LETS (PER C.M.M.)		
10	H. S.	59, W., M.	(1) 8.9	21.2	17,000	986,000	18 over 6 mo.	4.0	14.8	7,500	297,960	++	Diagnosed 1933 after coronary oc- clusion; therapy of venesections and roentgen radiation for 8 years with unsatisfactory control
11	E. S.	57, W., F.	(2) 7.3	21.4	14,950	648,560	11.0 over 12 mo.	5.85	15.4	9,000	368,000		Moderate resistance to P <sub>32</sub> therapy; venesections totaling 5,000 c.c. necessary to maintain equilibrium in addition to frequent P <sub>32</sub> ad- ministration
12	R. W.	40, W., M.	6.5	12.9	10,900	5,226,900	8.5 over 6 mo.	3.9	11.5	4,850	213,600	+	Two years of venesections totaled 9,250 c.c. before institution P <sub>32</sub> — 650 c.c. during therapy; note platelet response particularly
			7.18	15.4	29,000	1,959,000	6.1 over 2½ yr.	7.4	16.6	26,000	1,240,000	+++	Seen at very infrequent intervals; principal complaint was pruritus; small dosage P <sub>32</sub> would control this symptom satisfactorily for 2- to 4-month periods; hematologic studies too infrequent to evaluate effect on bone marrow; R.B.C. never over 7½ million; a rela- tively mild trait
13	J. H.	58, W., M.	7.8	11.0	45,000	1,884,000	9.1 over 10 mo.	6.0	8.9	12,500	593,140	+++	Patient expired from carcinoma of bladder; R.B.C., W.B.C., and platelets responded satisfactorily P <sub>32</sub> instituted after development of intolerance to phenylhydrazine therapy after two years. After a 5-month remission had throm- bosis of gastric vessels; remission for 1 year
14	A. K.	33	6.9	11.3	26,500	6,950,000	5.6 over 5 mo.	4.3	9.9	10,100	489,600	++++	

15	K. H.	47, W., F.	(1) 5.7 (2) 6.8	14.5 12.8	14,000 14,550	1,495,000 480,000	5.4 over 4 mo. 8.1 over 8 mo.	4.09 5.2 3.6	12.3 11.7 11.7	6,400 5,050 7,300	320,000 224,120 286,560	+++ +++ +++	Remissions lasting (1) 4 months and (2) 12 months induced; as the R.B.C. and hemoglobin levels rise, mental confusion increases Venesections and iotegen infusion over spleen for 2 years failed to control adequately R.B.C., W.B.C., or platelet elevations resulting in repeated episodes of thrombosis of arm and leg vessels; remission 1 year with P <sub>32</sub> therapy (Fig. 3)
16	W. A. R.	60, W., M.	7.9	12.8	24,700	1,249,720	10.6 over 4 mo.						Vertigo and early morning headaches markedly relieved; normal hemogram required 5 months of therapy; remission for 9 months continues
17	C. A.	57, W., M.	7.3	21.4	22,800	1,087,800	8.0 over 5 mo.	4.1	13.2	7,700	385,480	+++	In 1942 very small doses of radioactive strontium and phosphorus had no appreciable effect; in 1942-1943 venesections totaling 14,000 c.c. did not maintain the hematologic equilibrium (Fig. 4); normal hemogram in 3 months after therapy began; remission of 20 months; note platelets before and after
18	R. C.	48, W., F.	7.3	13.5	20,000	6,127,000	4.7 over 2 mo.	4.63	12.7	4,950	842,660	+++	Four years of therapy with venesections failed to control hematology picture satisfactorily; since 1941 remissions (1) lasting 12 months, (2) 17 months, and (3) 7 months have been induced with series of P <sub>32</sub> therapy (Fig. 2)
19	R. S.	73, W., F.	(1) 7.0 (2) 7.3 (3) 7.6	14.2 15.0 18.8	45,000 15,600 6,200	4,140,800 1,036,600 730,000	16.9 over 5 mo. 7.0 9.6 over 10 mo.	3.0 3.6 5.1	11.0 12.4 12.1	5,000 3,790 6,050	522,720 265,300 466,240	+++ +++ +++	Four months of excellent clinical improvement continues; hematology response gradually approaches normal
20	M. D.	65, W., F.	8.5	17.5	20,150	1,260,000	14.0 over 2 mo.	7.0	11.1	9,650	327,600	+++	

TABLE III. RADIOACTIVE PHOSPHORUS THERAPY IN CHRONIC LYMPHATIC LEUCEMIA

CASE	PATIENT	AGE, RACE, AND SEX	ONSET OF THERAPY					ESTIMATED DURATION OF DISEASE BEFORE P <sub>32</sub> THERAPY	DURATION OF DISEASE AFTER P <sub>32</sub> THERAPY	P <sub>32</sub> DOSE (MC.)	REMARKS
			W.B.C. (PER C.M.M.)	PER CENT OF LYMPHOCYTES	R.B.C. (PER C.M.M.)	HEMOGLOBIN (GM. %)	PLATELETS (PER C.M.M.)				
1	L.R.	57, W., M.	93,000	92	2.2	9.0	150,480	6 mo.	3 wk.	3.5 (o) over 14 days	Developed herpes zoster resistant to roentgen radiation during terminal phase; P <sub>32</sub> therapy effected symptomatic improvement; no significant hematologic effect
2	T.K.	51, W., M.	132,000	91	2.9	11.3	467,200	1 yr.	4 mo.	9.5 I.V. over 6 wk.	Lymphadenopathy and splenomegaly diminished significantly; W.B.C. 4 mo. later, 9,150 (with 77% lymphocytes); R.B.C., 1,980,000; platelets, 166,000; expired 2 weeks later
3	H.W.	53, W., M.	162,000	89	5.1	12.8	245,760	2½ yr.	1½ yr.	15.8 I.V. over 12 mo. period	Slight diminution in lymphadenopathy; total W.B.C. ranged from 80 to 100,000 while on therapy; R.B.C., 3,300,000 to 4,000,000; hemoglobin, 9.2 to 11.6; pruritis of leucemia cutis adequately controlled
4	G.V.	63, W., M.	3,500	42	3.1	10.2	161,200	2 yr.	7 mo.	4 I.V.	Resistant to roentgen radiation; pruritus of leucemia cutis relieved with P <sub>32</sub> therapy; lymphadenopathy decreased; no change in hematologic picture except progressive anemia to terminus
5	A.F.	53, W., M.	4,250	50	2.9	8.8	69,840	2 yr.	9 mo.	2.7 I.V. over 4 mo.	Coincident type; roentgen radiation fast; no significant hematologic or clinical improvement; progressive downhill course
6	G.W.	65, W., M.	126,000	94	3.48	8.1	313,200	?	1 yr.	3 I.V.	Resistant to roentgen radiation; P <sub>32</sub> had no significant results

7	B. C.	40, W., M.	136,000	93	37	10.7	131,280	?	5 yr.	17 I.V. over 3 mo. period	Received courses of $P^{32}$ therapy in 1910 and 1913,* accomplishing satisfactory remission; January to March, 1915, received 17 mc. $P^{32}$ ; lymphadenopathy decreased; June, 1915, W.B.C., 16,000; R.B.C., 2.8; platelets, 5,000; expired Nov. 18, 1915; received only $P^{32}$ over 5 year period
8	C. C.	57, W., M.	82,000	90	31	9.2	379,200	?	1 yr.	9 I.V. over 12 mo.	Diagnosis of carcinoma of sigmoid verified by biopsy, coexistent with chronic lymphatic leucemia; the leucemia held under excellent control with $P^{32}$ therapy; patient died of carcinoma with metastases to liver and lung; 1 week before death, W.B.C., 18,000 (62% lymphocytes); R.B.C., 3.0 (Fig. 6) developed roentgen radiation for 2 years; instituted; maintained W.B.C. between 15,000-30,000; platelets fell to 28,000; expired following intra-cranial hemorrhage, confirmed by post mortem examination
9	W. S.	15, W., M.	62,000	82	1.7	15.4	399,810	4 yr.	1 yr.	6 over 8 mo.	Received roentgen radiation for 2 years; developed marked hypersensitivity; $P^{32}$ instituted; maintained W.B.C. between 15,000-30,000; platelets fell to 28,000; expired following intra-cranial hemorrhage, confirmed by post mortem examination
10	W. C.	55, W., M.	90,000	96	3.3	9.1	93,210	6 mo.	21 mo. (Still under observation)	6 I.V. under 4 I.V.	$P^{32}$ continues to control leucemic process very satisfactorily; developed severe hemolytic anemia which was corrected with splenectomy (Fig. 5); therapy continued
11	C. A. S.	77, W., M.	12,700	50	1.8	10.9	192,360	10 mo.	9 mo.	11 I.V.	The leucemic process under excellent control with roentgen radiation; leucemia cutis developed and pustules was resistant to roentgen radiation; relieved with $P^{32}$ therapy

\*By Dr. John H. Lawrence, University of California

TABLE IV. RADIOACTIVE PHOSPHORUS THERAPY IN LEUCOSARCOMA

CASE	PATIENT	AGE, RACE, AND SEX	ONSET OF THERAPY					ESTIMATED DURATION OF DISEASE BEFORE P <sub>32</sub> THERAPY	DURATION OF DISEASE AFTER P <sub>32</sub> THERAPY	P <sub>32</sub> DOSAGE (MC.)	REMARKS
			W.B.C. (PER C.MM.)	PER CENT OF LYMPHO-BLASTS	R.B.C. (MILLIONS PER C.MM.)	HEMOGLOBIN (GM. %)	PLATELETS (PER C.MM.)				
1	N.B.	3, W., F.	939,000	85	3.2	8.0	259,200	3 wk.	5 wk.	7 over 4 days initially; 6 one mo. later	Reduction in W.B.C., 939,000 to 7,000 with definite improvement clinically; remission lasted 18 days; second elevation of W.B.C. to 485,000 was not effected by second course of P <sub>32</sub> (Fig. 7)
2	N.M.	5, W., F.	11,800	67	1.5	4.6	21,280	?	3 wk.	2	No change noted
3	M.S.	8, W., F.	72,000	94	21	5.2	16,360	8 wk.	6 wk.	4 over 7 days	600 r. units failed to have any appreciable effect on the blood picture; after 10 days P <sub>32</sub> instituted; W.B.C. fell from 200,000 to 11,000 in 10 days; terminal roentgen radiation ineffective; expired following massive gastrointestinal hemorrhage
4	M.B.	2, W., F.	102,000	89	2.6	6.5	189,600	6 wk.	3 wk.	2 (o)	Precipitous fall in W.B.C. from 102,000 to 5,500 in 2 days; continued fall to 200 at terminus; marked cervical adenopathy unaffected; 100 r. units to cervical nodes day before death
5	J.W.	7, W., M.	159,000	70	2.9	9.1	167,440	2 mo.	2 mo.	5 over 5 days	Gradual decline of W.B.C. over 2 weeks to 10,350; excellent clinical and hematologic remission for 4 weeks
6	J.D.	8, W., M.	75,000	88	4.5	12.4	336,608	6 mo.	2 mo.	3	Temporary remission of 4 weeks induced; received roentgen radiation during last 2 weeks of life





TABLE V. RADIOACTIVE PHOSPHORUS THERAPY IN MYELOGENOUS LEUCEMIA

CASE	PA- TIENT	AGE, RACE, AND SEX	ONSET OF THERAPY					ESTI- MATED DURA- TION OF DISEASE BEFORE P <sub>32</sub> THERAPY	DURATION OF DISEASE AFTER P <sub>32</sub> THERAPY	P <sub>32</sub> DOSAGE (MG.)	REMARKS
			W.B.C. (PER C.MM.)	PER CENT OF MYELO- CYTES	R.B.C. (MIL- LIONS PER C.MM.)	HEMO- GLOBIN (G.M. %)	PLATE- LETS (PER C.MM.)				
1	J. D.	46, W, M.	193,000	91 (Baso- philic myelo- cytes)	2.9	7.2	123,060	3 wk.	4 days	4.5 (1 dose)	Diagnosis: Acute basophilic leu- cemia; no improvement clin- ically or hematologically; ex- pired 4 days after therapy; post- mortem examination confirmed clinical impression Received intensive roentgen radia- tion during preceding 2 years which had failed to control the peripheral blood picture or the marked splenomegaly adequate- ly; progressive increase in W. B.C. to terminus; P <sub>32</sub> had no ef- fect on spleen size Peripheral W.B.C. maintained be- tween 3 to 5,000 and R.B.C. 3.5 to 5.0 satisfactorily; spleen failed to diminish in size ade- quately, therefore roentgen radi- ation totaling 800 r. given over the spleen; due to limited sup- ply P <sub>32</sub> , roentgen radiation used entirely for last year of life After three months of P <sub>32</sub> therapy there was a flare-up of an old osteomyelitis of mandible; 400 r. units given over adjacent mod- erate lymphadenopathy; W.B.C. ranged 8 to 20,000; R.B.C. 3.3 to 4.5; hemoglobin 9.2 to 12.5 Gm.; platelets fell to 33,000 terminally; splenomegaly ade- quately controlled
2	E. S.	24, W, M.	474,000	95	2.34	4.7	56,160	2½ yr.	26 days	5 (1 dose)	
3	W. C. W. 35, M.	W, M.	173,000	75	3.5	12.8	653,380	6 mo.	2 yr.	26.6 over 1-year period	
4	E. C.	51, W, M.	70,000	38	3.7	11.4	1,159,740	12 mo.	5 mo.	19 over 5 months	

5	J. J.	36, W., M.	189,000	38	2.9	9.1	560,240	15 mo.	5 mo.	Radioactive		Neither radioactive strontium nor phosphorus had appreciable clinical or hematologic response; roentgen radiation to spleen brought about temporary remission; expired 6 months after onset of therapy
										strontium, 1.1 over 3 weeks; P <sub>32</sub> 11.5 over 4 weeks		
6	R. M.	61, W., F.	30,300	25	3.58	7.0	436,760	12 mo.	26 mo.	24 over 10 months		P <sub>32</sub> therapy maintained a mean W.B.C. of approximately 16,000; R.B.C., 3.8; hemoglobin, 10.5 Gm. for 18 months; splenomegaly was principal source of symptoms; roentgen radiation therapy for last 10 months of life
7	C. R.	51, W., F.	54,000	43	3.9	10.2	555,220	16 mo.	10 mo.	38 (o) 7 months		Developed hypersensitivity to roentgen radiation after 16 months; W.B.C. not adequately controlled (mean W.B.C., 95,000); R.B.C., 3.5 to 4.6; hemoglobin, 9.8 to 12.3 Gm.; splenomegaly controlled; remission 10 months
8	L. E. T.	33, W., M.	125,000	47	4.4	13.0	674,000	3 yr.	10 mo.	8½ over 2½ months		No previous therapy; excellent remission with control of splenomegaly 1 year; W.B.C. 22 to 54,000; R.B.C., 4.2 to 4.6; hemoglobin, 12.5 to 13 Gm.; now receiving second course of therapy
9	G. M.	35, W., M.	294,000	54	3.3	6.9	893,700	14 yr.	7 mo.	10 over 7 months		Unable effectively to lower W.B.C. below 100,000; no significant effect on splenomegaly; R.B.C., 3 to 4.2; hemoglobin, 6.0 to 10.5 Gm.
10	A. W.	62, W., F.	103,000	31	3.6	10.5	796,400	3 yr.	1 yr.	17 over 1 year		Developed severe reactions to roentgen radiations after 2½ years; Fowler's solution (4 months) ineffective; P <sub>32</sub> held satisfactory hematologic remission; W.B.C., 8 to 50,000; R.B.C., 3.2 to 3.9; hemoglobin 8 to 11.4 Gm.; no effect on marked splenomegaly (Fig. 8)

TABLE V—CONT'D

CASE	P.A. TIENT	AGE, RACE, AND SEX	ONSET OF THERAPY				ESTI- MATED DURATION OF DISEASE BEFORE P <sub>32</sub> THERAPY	DURATION OF DISEASE AFTER P <sub>32</sub> THERAPY	P <sub>32</sub> DOSAGE (MC.)	REMARKS
			W.B.C. (PER C.MM.)	PER CENT OF MYELO- CYTES	R.B.C. (MIL- LIONS PER C.MM.)	HEMO- GLOBIN (GM. %)				
11	M.L.	55, W., F.	297,000	63	2.9	7.7	623,280	3 yr.	Continues 12 over 4 weeks 1 yr.	Reduced W.B.C. to 118,000 in 3 weeks; elevation of R.B.C. from 2.9 to 3.8; hemoglobin, 7.7 to 10.1 Gm.; clinically, little change; roentgen radiation instituted
12	J.B.	41, W., M.	261,000	78	3.9	9.5	435,500	2 yr.	22 mo. 14 over 22 months	P <sub>32</sub> has maintained W.B.C. mean of 75,000 over 22-month period; R.B.C., 4.5, and hemoglobin, 12 Gm., without transfusions; splenomegaly controlled satisfactorily
13	R.M.	49, W., M.	100,000	42	3.0	8.9	1,043,280	3 yr.	24 mo. 37 over 22 months	Developed marked sensitivity to roentgen radiation after 3 years of successful control; P <sub>32</sub> therapy for 22 months has not satisfactorily controlled W.B.C. (averaged 175,000) and splenomegaly; R.B.C. mean 3.0, with 9.1 Gm. of hemoglobin
14	B.R.	52, W., F.	176,000	43	3.7	7.5	677,040	2 yr.	9 mo. 13 over 3 months	Developed marked intolerance to both roentgen and urethane therapy; P <sub>32</sub> gradually reduced W.B.C. with corresponding rise in R.B.C., but splenomegaly not appreciably effected

TABLE VI. RADIOACTIVE PHOSPHORUS THERAPY IN MONOCYTIC LEUCÆMIA

CASE	PATIENT	AGE, RACE, AND SEX	ONSET OF THERAPY				DURATION OF DISEASE AFTER INSTITUTION OF P <sup>32</sup> THERAPY	REMARKS
			W.B.C. (PER G.M.M.)	PER CENT OF MONOCYTES	R.B.C. (MILLIONS PER G.M.M.)	HEMOGLOBIN (GM. %)		
1	D. M.	68, W, F.	26,800	89 (monocytes)	3.65	6.1	21 mo.	A total of 25 mc. (o) over 21-month period effected a moderate improvement clinically; the spleen diminished in size during the first 12 months of therapy; the total W.B.C. was not appreciably altered; there was a slow and progressive rise to a terminal W.B.C. of 284,000 with 97% monoblasts
2	J. F. B.	3, W, M.	39,550	. 67 (monoblasts)	4.16	11.9	6 wk.	2 mc. P <sup>32</sup> orally was followed by a fall in the W.B.C. from 39,550 to 6,100 in 7 days
3	C. H.	5, W, F.	526,000	96 (monoblasts)	2.7	6.3	7 wk.	5 mc. P <sup>32</sup> followed by fall in W.B.C. to 8,600 in 21 days; second injection of 1½ mc. had no appreciable effect on the blood picture
4	A. K.	45, W, F.	3,950	45 (monoblasts)	2.8	10.5	4 mo.	25 mc. P <sup>32</sup> orally over 4-month period failed to achieve any beneficial effect; there was a steady rise to a terminal W.B.C. of 171,000
5	E. G.	61, W, F.	30,300	70 (monoblasts)	3.03	11.2	4 wk.	2 mc. P <sup>32</sup> (I.V.) had no effect over a 4-week observation period
6	E. S.	54, W, M.	165,000	81 (monoblasts)	2.5	8.5	4 days	0.1 mc. radioactive strontium (o) ineffective; steady rise in W.B.C. followed by 4.2 mc. P <sup>32</sup> ; continued rise in W.B.C. to 308,000 and terminus 4 days later
7	R. C.	43, W, F.	34,900	12 (monoblasts)	2.97	10.2	4 days	4.8 mc. P <sup>32</sup> (I.V.) over 2-day period effected no results; steady rise in W.B.C. to 69,000 4 days later
8	A. W.	59, W, M.	127,000	95 (monoblasts)	2.1	7.4	4 wk.	5 mc. P <sup>32</sup> (I.V.); no appreciable change in hematologic picture except progressive anemia and thrombocytopenia; no effect on W.B.C.
9	J. W.	32, W, M.	253,000	81 (monoblasts)	3.9	10.5	12 wk.	6 mc. (over 3 days); lymphadenopathy diminished; fall in W.B.C. from 253,000 to 88,000 in 14 days; W.B.C. 2½ months after therapy 4,100 (Fig. 10)
10	P. C.	38, W, M.	10,650	79 (monoblasts)	3.13	8.3	9 wk.	Received 5 mc. P <sup>32</sup> over six weeks; no significant clinical or hematologic change

TABLE VII. RADIOACTIVE PHOSPHORUS THERAPY IN MISCELLANEOUS DISEASES

CASE	PATIENT	AGE, RACE, AND SEX	DIAGNOSIS	P <sup>32</sup> DOSAGE (MC.)	SUBJECTIVE RESULTS	OBJECTIVE RESULTS	HEMATOLOGIC RESULTS
1	C. M. C.	58, W., M.	Carcinoma of prostate (biopsy) with metastasis to cranium, pelvis, lumbar spine,	11 over 3-month period	Excellent transient relief of bone pain	No change roentgenologically	R.B.C. fell from 3.5 to 1.4 million and W.B.C. from 8,600 to 1,850 after 2 months of therapy
2	A. H.	25, W., M.	Astrocytoma of cerebellum with metastasis to spleen, bone marrow	17 over 3½-month period	Temporary relief of bone pain	None	Moderate leucopenia (2,600) developed during therapy
3	J. H.	19, W., M.	Retroperitoneal sarcoma 5 with pulmonary metastasis	5	None	None	None
4	E. N.	68, W., M.	Exfoliative dermatitis	7½ over 4-month period	Pruritus markedly relieved	Dermatitis improved significantly	Moderate diminution of platelets, R.B.C., and granulocytes during therapy (Fig. 1)
5	S. F.	54, W., F.	Multiple myeloma	3 (patient was in terminal state)	None	None	None
6	J. W.	37, W., M.	Multiple myeloma	11.5 over 7-month period	Bone pain satisfactorily controlled for 7 months; roentgen radiation used terminally due to inadequate supply P <sub>32</sub>	None	None
7	S. A. L.	48, W., M.	Multiple myeloma	15 (o) over 6-week period	Deep bone pain partially relieved for 7 to 10 days following each administration	None	None
8	L. L. V.	43, W., M.	Mycosis fungoides	5.3 (o) over 6-week period	None—progressive downhill course to terminus 2 months after onset of therapy	None	None
9	E. D.	50, W., F.	Carcinoma of breast with metastasis to vertebrae, ribs, liver, and lungs	5.0	No significant relief from bone pain	None	None
10	C. J.	35, W., M.	Seminoma of testicle with generalized metastasis	5.0	No relief from bone pain	None—no roentgenologic evidence of regression of bone lesions; no regression of subcutaneous nodules	None
11	E. M.	48, W., M.	Multiple myeloma	9.5 over 3 months	Clinical status stationary for 6 months	No change roentgenologically	Transient leucopenia (3,350 per c.mm.) developed during therapy

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# REFERENCES

1. Senn, N.: The Therapeutical Value of the Roentgen Ray in the Treatment of Leukemias Summarized From a Series of Twenty-Five Cases, *Am. J. Roentgenol.* 12: 126, 1903.
2. Low-Beer, B. V. A., Lawrence, J. H., and Stone, R. S.: The Therapeutic Use of Artificially Produced Radioactive Substances, *Radiology* 39: 573, 1942.
3. Erf, L. A., and Lawrence, J. H.: Clinical Studies With the Aid of Radio-Phosphorus; Absorption and Distribution of Radio-Phosphorus in the Blood of, Its Excretion by, and Its Therapeutic Effect on, Patients With Polycythemia, *Ann. Int. Med.* 15: 276, 1941.
4. Erf, L. A., Tuttle, L. W., and Lawrence, J. H.: Clinical Studies With the Aid of Radio-Phosphorus. IV. The Retention in the Blood, the Excretion and the Therapeutic Effect of Radio-Phosphorus on Patients With Leukemia, *Ann. Int. Med.* 15: 487, 1941.
5. Fitz-Hugh, T., and Hodes, P. J.: Clinical Experience With Radio-Phosphorus in the Treatment of Certain Blood Dyscrasias, *Am. J. M. Sc.* 5: 662, 1942.
6. Kenney, J. M.: Radioactive Phosphorus as a Therapeutic Agent in Malignant Neoplastic Disease, *Cancer Research* 2: 130, 1942.
7. Craver, L. F.: Treatment of Leukemias by Radioactive Phosphorus, *Bull. New York Acad. Med.* 18: 259, 1942.
8. Warren, S.: The Therapeutic Use of Radioactive Phosphorus, *Am. J. M. Sc.* 209: 701, 1945.
9. Hall, B. E., Watkins, C. H., Hargraves, M. M., and Giffin, H. Z.: Radioactive Phosphorus in the Treatment of Polycythemia Vera: Results and Hematologic Complications, *Am. J. M. Sc.* 209: 712, 1945.
10. Erf, L. A.: Primary Polycythemias; Remissions Induced by Therapy With Radio-phosphorus, *Blood* 1: 202, 1946.
11. Erf, L. A.: Radiophosphorus as the Treatment of Choice in Primary Polycythemia, *Am. J. Med.* 1: 363, 1946.
12. Hoster, H. A., and Doan, C. A.: Studies in Hodgkin's Syndrome: IV. The Therapeutic Use of Radioactive Phosphorus, *J. LAB. & CLIN. MED.* 30: 678, 1945.
13. Reinhard, E. H., Moore, C. V., Bierbaum, O. S., and Moore, S.: Radioactive Phosphorus as a Therapeutic Agent, *J. LAB. & CLIN. MED.* 31: 107, 1946.
14. Davison, R. A., Zollinger, R. W., and Curtis, G. M.: The Fractionization of the Blood Iodine, *J. LAB. & CLIN. MED.* 27: 643, 1942.
15. Gibson, J. G., and Evelyn, K. A.: Clinical Studies of the Blood Volume; Adaptation of the Method to the Photoelectric Microcolorimeter, *J. Clin. Investigation* 17: 153, 1938.
16. Rourke, M. D., and Ernstene, A. C.: Method for Correcting the Erythrocyte Sedimentation Rate for Variations in the Cell Volume Percentage of Blood, *J. Clin. Investigation* 8: 545, 1930.
17. Aebersold, P. C.: The Cyclotron: A Nuclear Transformer, *Radiology* 39: 513, 1942.
18. Hempelmann, L. A., Reinhard, E. H., Moore, C. V., Bierbaum, O. S., and Moore, S.: Hematologic Complications of Therapy With Radioactive Phosphorus, *J. LAB. & CLIN. MED.* 29: 1020, 1944.

# FOLIC ACID IN THE MAINTENANCE OF PERNICIOUS ANEMIA

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THE first report on the effect of folic (pteroylglutamic) acid in the treatment of macrocytic anemia appeared in November, 1945.<sup>1</sup> Since then numerous reports describing the results obtained in the treatment of pernicious anemia,<sup>2</sup> macrocytic anemia of nutritional deficiency,<sup>3</sup> sprue,<sup>4</sup> macrocytic anemia of pregnancy,<sup>5</sup> and macrocytic anemia of infancy<sup>6</sup> have appeared. In general, folic acid has been found to cause a hematologic improvement similar to or identical with that of liver extracts when given to patients with macrocytic anemia in relapse, as judged by the response of the reticulocytes, erythrocytes, white cells, and platelets. In sprue, in addition, there is a rapid subsidence of diarrhea and a marked improvement in intestinal absorption. One author, however, after study of a series of cases of pernicious anemia,<sup>9</sup> has concluded that folic acid does not produce as great a reticulocyte response or as complete return to normal of erythrocytes as occurs with liver extracts. Further, he concluded that a combination of liver extract and folic acid is more effective than either alone. These latter conclusions were based upon the observation that reticulocyte response to the combined therapy was greater than might have been expected in some of the cases and the increase in erythrocytes was somewhat more rapid than occurred with either alone. This study compared results obtained in different patients, rather than in the same patient, using as the basis for comparison successive periods of treatment with folic acid, with liver extract, and with a combination of the two. Because of the well-known variation of response in different patients, the validity of comparisons made in different individuals is doubtful, unless the number of comparisons made was sufficient to permit statistical evaluation.

Neurologic relapse has been observed during therapy with folic acid in patients with pernicious anemia, even when the blood was well maintained at normal levels and in the absence of macrocytosis.<sup>6-8</sup>

The study reported here was undertaken to determine the effectiveness of synthetic folic acid (pteroylglutamic acid)\* in maintenance therapy of pernicious and allied macrocytic anemia, and an attempt has been made to compare such treatment with liver extract therapy.

## I. MATERIAL AND METHODS

The study included 41 patients with proved macrocytic anemia. In 35, the results of folic acid therapy were compared with previous antipernicious anemia therapy with intra-

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muscular injections of crude and purified liver extracts, oral liver-stomach therapy (Extralin, Lilly) and stomach therapy (Ventriculin, Parke, Davis & Co.). Folic acid therapy was given to 6 patients who were in severe hematologic relapse.

The folic acid was administered parenterally to 25 patients and orally to 16 patients. The intramuscular route was used for all parenteral injections, except in one patient, who insisted upon intravenous administration. The parenteral dose was 50 mg. in 15 patients and 150 mg. in 9 patients. Intervals between injections of from two to six weeks were employed, although the usual interval was either three or four weeks. The duration of this interval was determined, whenever possible, by the previous intervals between injections of liver extract.

When given orally, folic acid was administered in daily doses of 10 to 40 mg.; the usual dose was either 10 or 20 milligrams. No patient in this series was treated with a daily oral dose of less than 10 milligrams.

There were 22 women and 19 men in the series; of these, 33 were white and 8 were Negro. The age of the patients ranged from 37 to 82 years, the average age being 62.4 years. Three patients developed macrocytic anemia following gastric resection and one patient had macrocytic anemia in association with syphilitic fibrosis of the stomach. The others were well-substantiated cases of addisonian pernicious anemia, as evidenced by the presence of macrocytic anemia, histamine-fast achlorhydria, response to liver extracts or stomach concentrate and, in some cases, the presence of combined system disease and megaloblastic hyperplasia of the bone marrow.

The incidence of degenerative diseases was high, as would be expected in a group of patients in this age range. In addition, 5 patients had syphilis and 2 had diabetes; 3 had had gastric resection, 2 for peptic ulcer and 1 for carcinoma of the stomach.

Because of the errors inherent in the determination of the erythrocyte counts and because the determinations made before the institution of folic acid therapy were performed by different technicians, it was arbitrarily decided that differences in the erythrocyte count of less than 300,000 per cubic millimeter would not be considered significant. A reduction of 300,000 to 500,000 cells per cubic millimeter was considered a moderate decrease; that of 500,000 per cubic millimeter or more was considered a marked decrease. Conversely, an increase of 300,000 cells per cubic millimeter or more was considered evidence of improvement.

In Tables I to IV the values for the erythrocyte count and hemoglobin level prior to folic acid therapy represent the average of many determinations over a long period of time. Values obtained at the end of the period of observation on folic acid therapy represent an average of at least three determinations. Hematocrit values were not regularly determined, either before or after the institution of folic acid therapy. The authors are cognizant of the fact that without hematocrit determinations definite conclusions concerning macrocytosis cannot be drawn.

All the patients were carefully examined, before and during the course of therapy with folic acid, for changes in deep tendon reflexes, diminution of vibratory sensation, astereognosis, muscle coordination, and the presence of positive Babinski and Romberg signs. An attempt was made to evaluate the nutritional status, as judged by weight and evidence of vitamin deficiency (glossitis, cheilosis, stomatitis, gingivitis). The patients were carefully and repeatedly questioned regarding subjective changes in their state of well-being.

## II. RESULTS

*A. Hematologic Observations.*—The patients were divided into two groups: (1) those studied six to twelve months and (2) those studied less than six months.

The first group, consisting of 32 patients, was studied six to twelve months, with an average of 10.7 months. Of these, 23 or 71.9 per cent, were maintained in a satisfactory hematologic state. Eighteen of the 23 (Table I) showed no

TABLE I. PATIENTS TREATED WITH FOLIC ACID WHO SHOWED NO SIGNIFICANT CHANGE IN ERYTHROCYTE LEVELS

CASE	AGE	SEX	COLOR	TIME ON F.A. (MO.)	DOSE OF F.A. (MG.)	INTER- VAL BE- TWEEN DOSES	ROUTE	AV. BLOOD VALUES FOR YEAR BEFORE F.A.		AV. BLOOD VALUES AFTER F.A.		CHANGE	
								R.B.C. ×10 <sup>6</sup>	Hb. (GM.)	R.B.C. ×10 <sup>6</sup>	Hb. (GM.)	R.B.C. ×10 <sup>6</sup>	Hb. (GM.)
Group I—Treated for Six to Twelve Months													
1	82	M	W	12	150	3 wk.	I.V.	3.60	12.7	3.41	11.6	-0.19	-1.1
2	52	F	W	12	150	3 wk.	I.M.	4.65	13.6	4.38	13.1	-0.27	-0.5
3	67	M	W	10	150	3 wk.	I.M.	4.32	13.9	4.51	13.6	+0.19	-0.3
4	50	F	W	11	150	4 wk.	I.M.	4.51	13.9	4.39	13.4	-0.12	-0.5
5	72	F	W	11	150	4 wk.	I.M.	4.38	14.0	4.12	13.8	-0.26	-0.2
6	48	F	W	11	50	2 wk.	I.M.	4.03	12.5	3.99	12.3	-0.40	-0.3
7	53	F	W	11	40	3 wk.	I.M.	4.65	13.9	4.40	13.8	-0.25	-0.1
8	63	F	W	11	50	3 wk.	I.M.	4.63	13.4	4.44	13.6	-0.18	+0.2
9	81	M	W	12	50	4 wk.	I.M.	4.48	13.4	4.69	13.4	+0.21	0
10	57	F	W	12	50	4 wk.	I.M.	4.20	13.9	4.45	13.8	+0.25	-0.1
11	56	M	W	7	50	4 wk.	I.M.	4.33	14.9	4.14	13.9	-0.19	-1.0
12	67	M	W	10	20	Daily	Oral	4.25	13.6	4.31	12.9	+0.60	-0.7
13	64	F	W	12	10	Daily	Oral	4.09	12.8	3.97	12.3	-0.12	-0.5
14	73	M	W	12	10	Daily	Oral	4.43	14.4	4.26	14.4	-0.17	0
15	57	F	W	12	10	Daily	Oral	4.44	12.9	4.73	14.0	+0.29	+1.1
16	65	F	W	7	10	Daily	Oral	4.56	14.0	4.85	14.7	+0.29	+0.7
17	78	F	W	7	10	Daily	Oral	4.52	13.1	4.45	13.3	-0.07	+0.2
18	74	M	W	6	10	Daily	Oral	4.48	13.8	4.51	13.9	+0.03	+0.1
Group II—Treated Less Than Six Months													
19	59	F	W	3	50	4 wk.	I.M.	4.55	13.4	4.54	13.4	-0.01	0
20	46	F	C	4	10	Daily	Oral	4.48	12.5	4.26	10.2	-0.22	-2.3

significant change in erythrocyte count or in hemoglobin level, in comparison with previous findings obtained following therapy with liver extract; in three cases, improvement was manifested (Table III). One patient (Case 31, Table III) was not in good hematologic remission before institution of folic acid therapy. She was a 51-year-old white woman who was extremely sensitive to substances in liver extract and who had been maintained on nine to fifteen capsules daily of an oral liver-stomach preparation, Extralin B (Lilly), on which therapy normal blood values were not obtained. Another patient (Case 32, Table III) had also been maintained on Extralin B, because of marked sensitivity to intramuscular injections of liver extract, and her erythrocyte count and hemoglobin level were well below normal when folic acid therapy was instituted. Two patients in this group were started on folic acid therapy when in relapse; they had received no liver extract therapy for several months prior to this time (Table IV). These patients showed satisfactory clinical and hematologic improvement.

In 2 of this group of 32 patients a moderate decrease in erythrocyte count occurred, while in 7 a marked decrease developed, as already defined (Table II).

The second group consisted of nine patients studied for one to five months (average, 3.3 months). None of these showed any significant decrease in erythrocyte count. Two showed no appreciable change (Table I) and 3 showed an increase in erythrocyte count, as compared with previous liver extract therapy (Table III). The other four were started on folic acid therapy when in re-

TABLE II. PATIENTS TREATED WITH FOLIC ACID WHO SHOWED MODERATE (300,000 TO 500,000 PER CUBIC MILLIMETER) OR MARKED (OVER 500,000 PER CUBIC MILLIMETER) DECREASE IN ERYTHROCYTE LEVELS

CASE	AGE	SEX	COLOR	TIME ON F.A. (MO.)	DOSE OF F.A. (MG.)	INTER- VAL BE- TWEEN DOSES	ROUTE	AV. BLOOD VALUES FOR YEAR BEFORE F.A.		AV. BLOOD VALUES AFTER F.A.		CHANGE	
								R.B.C.	HB.	R.B.C.	HB.	R.B.C.	HB.
								×10 <sup>6</sup>	(GM.)	×10 <sup>6</sup>	(GM.)	×10 <sup>6</sup>	(GM.)
Group I—Treated for Six to Twelve Months Moderate Decrease in Erythrocyte Count (300,000 to 500,000 Per Cubic Millimeter)													
21	51	F	C	11	150	4 wk.	I.M.	4.53	14.0	4.11	13.4	-0.42	-0.6
22	58	M	C	11	50	4 wk.	I.M.	4.19	12.9	3.85	12.7	-0.34	-0.2
Group I—Treated for Six to Twelve Months Marked Decrease in Erythrocyte Count (Over 500,000 Per Cubic Millimeter)													
23	59	M	W	11	150	4 wk.	I.M.	3.76	13.4	2.55	9.8	-1.21	-3.6
24	75	M	W	12	150	6 wk.	I.M.	4.15	13.9	3.56	12.5	-0.59	-1.4
25	70	F	W	9	150	6 wk.	I.M.	4.48	13.8	3.75	12.8	-0.73	-1.0
26	61	F	W	12	50	3 wk.	I.M.	4.73	13.8	3.43	10.8	-1.30	-3.0
27	80	F	W	12	50	3 wk.	I.M.	4.19	13.4	3.48	12.2	-0.71	-1.2
28	37	M	C	12	50	4 wk.	I.M.	4.34	14.2	3.39	13.6	-0.95	-0.6
29	57	M	C	8	50	4 wk.	I.M.	4.79	13.3	4.11	11.3	-0.68	-2.0

TABLE III. PATIENTS TREATED WITH FOLIC ACID WHO SHOWED AN IMPROVEMENT IN ERYTHROCYTE LEVELS (MORE THAN 300,000 PER CUBIC MILLIMETER)

CASE	AGE	SEX	COLOR	TIME ON F.A. (MO.)	DOSE OF F.A. (MG.)	INTER- VAL BE- TWEEN DOSES	ROUTE	AV. BLOOD VALUES FOR YEAR BEFORE F.A.		AV. BLOOD VALUES AFTER F.A.		CHANGE	
								R.B.C.	HB.	R.B.C.	HB.	R.B.C.	HB.
								×10 <sup>6</sup>	(GM.)	×10 <sup>6</sup>	(GM.)	×10 <sup>6</sup>	(GM.)
Group I—Treated for Six to Twelve Months													
30	76	F	W	11	50	4 wk.	I.M.	4.76	14.9	5.07	15.1	+0.31	+0.2
31	51	F	W	12	10	Daily	Oral	3.71	12.2	4.04	12.7	+0.33	+0.5
					40								
32	77	F	W	0	20	Daily	Oral	3.04	10.6	4.16	11.4	+1.12	+0.8
Group II—Treated for Less Than Six Months													
33	69	F	W	5	50	2 wk.	I.M.	4.48	13.6	4.92	13.4	+0.44	-0.2
34	76	F	W	1	45	2 wk.	I.M.	4.15	12.8	4.59	13.1	+0.44	+0.3
35	78	M	W	3	20	Daily	Oral	4.15	12.9	4.71	12.8	-0.56	-0.1

lapse without having had recent previous liver extract therapy (Table IV). A satisfactory hematologic remission ensued in every case. One patient (Case 40, Table IV), at the beginning of treatment, had an erythrocyte count of 340,000 per cubic millimeter; hemoglobin, 1.4 Gm. per 100 ml. of blood; leucocyte count, 1,200 per cubic millimeter; hematocrit, 4.0; mean corpuscular volume, 117.5 cubic microns; mean corpuscular hemoglobin, 41.2 micromicrograms; mean corpuscular hemoglobin concentration, 35 per cent. These values are the average of determination done in triplicate. In our experience they represent the most severe anemia ever seen in pernicious anemia from which the patient recovered.

It should be noted that in the entire series of 41 patients, only 9 had a significant decrease in the erythrocyte count as compared with previous liver

TABLE IV. PATIENTS TREATED WITH FOLIC ACID FROM RELAPSE

CASE	AGE	SEX	COLOR	TIME ON F.A. (MO.)	DOSE OF F.A. (MG.)	INTER- VAL BE- TWEEN DOSES	ROUTE	BLOOD AT START OF F.A. THERAPY		AV. BLOOD VALUES AFTER F.A.		CHANGE	
								R.B.C. ×10 <sup>6</sup>	Hb. (GM.)	R.B.C. ×10 <sup>6</sup>	Hb. (GM.)	R.B.C. ×10 <sup>6</sup>	Hb. (GM.)
Group I—Treated for Six to Twelve Months													
36	47	F	C	11	20	Daily	Oral	1.00	3.9	3.90	10.9	+2.90	+7.0
37	76	F	W	11	20	Daily	Oral	3.10	11.9	1.24	13.6	+1.14	+1.7
Group II—Treated for Less Than Six Months													
38	52	M	C	5	50	4 wk.	I.M.	1.02	3.4	4.58	13.6	+3.56	+10.2
39	49	F	W	5	30 to 40	Daily	Oral	2.42	11.1	4.75	13.9	+2.33	+2.5
40	45	M	C	4	10	Daily	Oral	0.34	1.1	4.81	11.5	+4.47	+10.1
41	68	M	W	2	10	Daily	Oral	1.80	5.6	5.09	15.6	+3.29	+10.0

extract therapy (Table II). But, in all these cases, injections were at intervals of three to six weeks; in 5 patients the individual dose was 50 mg., while in the other 4 the dose given was 150 milligrams. The size of the individual dose was not correlated with the severity of the relapse in this group. None of the patients receiving oral therapy had hematologic relapse.

On the other hand, 6 patients of the entire series of 41 showed definite improvement in erythrocyte values as compared with previous liver extract therapy (Table III). Of these, 3 were receiving daily oral medication, 2 were receiving either 45 or 50 mg. at intervals of two weeks, and only 1 was given 50 mg. at intervals greater than two weeks. It should be noted that with the exception of the last patient, those showing improvement were either on daily oral therapy or were receiving intramuscular injection at intervals of two weeks, the shortest period used in this study.

Of the 6 patients showing improvement, 2 (Cases 31 and 32, Table III) had blood levels definitely below normal values at the time of institution of folic acid therapy, while 2 others (Cases 34 and 35, Table III) had blood levels suggestively lower than normal. The improvement obtained with folic acid therapy, therefore, probably indicates that the patients had not been receiving enough liver extracts previously or, as in the case of the patients (Cases 31 and 32), who had been on oral liver extract therapy, were not absorbing the liver extract efficiently enough to maintain normal blood levels.

Of the patients showing hematologic relapse, one case is of particular interest. He was a 59-year-old Italian who had a diagnosis of pernicious anemia established many years before and who had been treated regularly with intramuscular liver extract injections. Recent liver extract therapy had not maintained the blood at normal levels, possibly because of insufficient dosage at insufficiently frequent intervals. When transferred to parenteral folic acid therapy (150 mg. at four-week intervals) there was a further marked drop in erythrocytes and hemoglobin, although the patient stated that he felt well (Fig. 1). He was admitted to the hospital for study, and a reticulocyte count made on the fifth day after the last dose of folic acid revealed a value of 13 per cent

(Fig. 1). Without further treatment the reticulocytosis subsided and there was an increase in red count and hemoglobin. At the end of four weeks, however, the erythrocytes and hemoglobin had again decreased. The rapidity of decrease in the erythrocyte count suggested the presence of a hemolytic process. Determinations of icterus index, serum bilirubin, van den Bergh, and quantitative urine urobilinogen did not support this idea, however. Studies of fecal urobilinogen were not made. It seems likely that each injection of folic

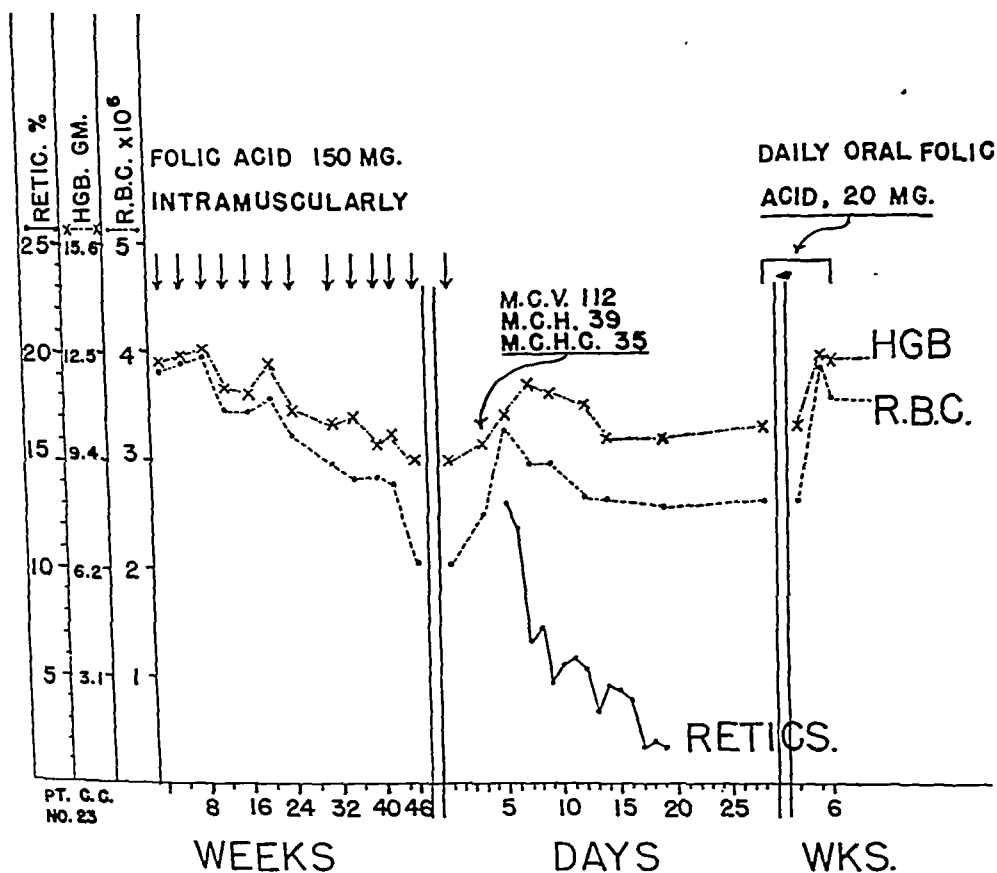


Fig. 1.—Patient (Case 23, Table II) showing hematologic relapse on folic acid therapy, temporary improvement after single injection of folic acid, and improvement after therapy with daily oral doses of folic acid.

acid in this patient caused a characteristic reticulocyte response with increase in erythrocytes and hemoglobin, but this response had ended and the patient had relapsed a little further by the end of each four-week interval. Thus, the course was probably one of alternate responses and relapses, with each relapse carrying the blood values a little lower than before. Such a finding is not surprising in view of our knowledge of folic acid excretion. Steinkamp and co-workers<sup>9</sup> have shown that a large part of administered folic acid is excreted in a relatively short time. Welch and Heinle<sup>10</sup> have been able to demonstrate little, if any, "storage" of folic acid from study of urinary excretion.

When the patient was placed on oral therapy with folic acid, 10 mg. daily (Fig. 1), there was an improvement in the erythrocyte count to a level maintained when the patient was receiving liver extract. Folic acid, however, like the previous liver extract therapy, was unable to produce a normal erythrocyte count in this patient. Bone marrow studies, including fixed sections of particles obtained by sternal aspiration, did not reveal an unusual amount of fat in the marrow.

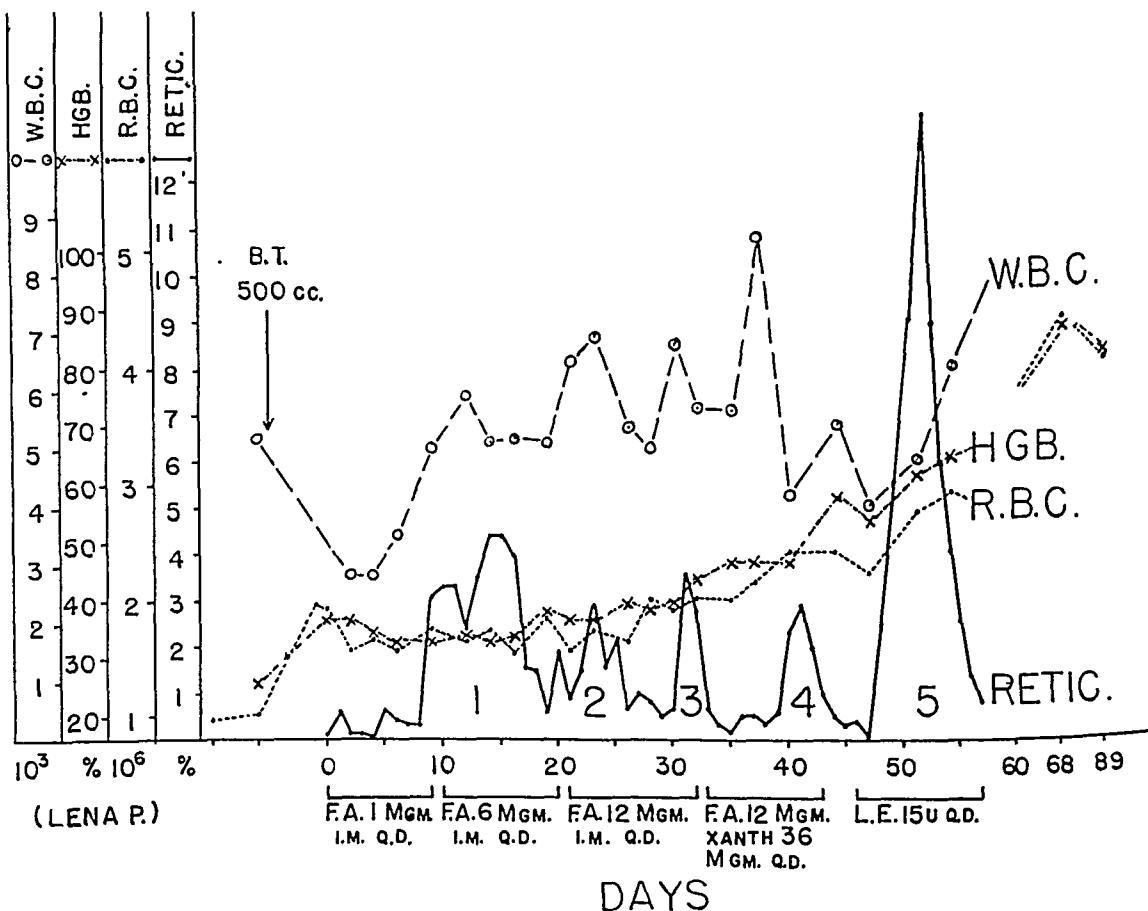


Fig. 2.—Patient L. P. 100% hemoglobin=15.6 Gm. per 100 milliliter. Xanth.=xanthopterin. L.E.=Lederle solution of liver extract, 15 units per milliliter. B.T.=Blood transfusion.

Outright failure of response to folic acid has not been reported. One patient observed by us showed very little response to doses of folic acid which have provided a good, if not theoretically maximal, response in all our other patients. This patient was a 46-year-old colored woman with a macrocytic anemia, histamine-fast achlorhydria, and megaloblastic hyperplasia of the bone marrow. During a period of ten days (Fig. 2) she was given daily intramuscular injections of 1 mg. of folic acid with a delayed and very small reticulocyte response (1, Fig. 2). During a second period of eleven days the daily intra-

muscular dose of folic acid was increased to 6 mg. with a second, delayed, very small reticulocyte response (2, Fig. 2). During a third period of twelve days, the daily intramuscular dose of folic acid was increased to 12 mg. with another very small reticulocyte response (3, Fig. 2). During a fourth period of thirteen days the daily intramuscular injections of 12 mg. of folic acid were continued with the addition of daily oral doses of 36 mg. of xanthopterin. A fourth small reticulocyte response (4, Fig. 2) followed. During this entire period of forty-six days there was only a very slight increase in erythrocytes and hemoglobin. She was then given daily intramuscular injections of 15 units of purified liver extract (Lederle, 15 units per milliliter), and on the fifth day after the start of this therapy a theoretically maximal reticulocyte response occurred (5, Fig. 2) and there was a rapid increase in erythrocytes and hemoglobin. The folic acid used in treating this patient was assayed with *Lactobacillus casei* and found to be as potent as labeled. Unfortunately, it was not possible to carry out studies of urinary excretion in this patient.

The reason for failure of response to a dose of folic acid sufficient to produce a good response in all of our other patients is not clear nor is there an obvious explanation for the prompt response to liver extract after failure of folic acid. We have not encountered any other such patients to date, but a similar failure in two patients of Shukers has been referred to by Darby.<sup>11</sup>

The patient was continued on liver extract therapy for ten months, at the end of which time the erythrocyte count was 4,510,000 per cubic millimeter and the hemoglobin level was 12.6 Gm. per 100 milliliters. At that time she was transferred to folic acid therapy and given daily oral doses of 10 milligrams. During a period of five months on folic acid therapy there has been no significant change in blood values, body weight, or symptoms.

*B. General Clinical Observations.*—The majority of the patients noted no difference in subjective sense of well-being. The few who did not feel as well were among those receiving intermittent intramuscular injections with decrease in blood values. These patients observed that they felt well for a short time after each injection of folic acid but that the effect did not last as long as with liver extract injections. There were a few patients, particularly those receiving oral medication, who claimed subjective improvement, especially an increase in appetite, after institution of folic acid therapy. No evidence of associated vitamin deficiencies was observed in any of these patients. Body weight was maintained in all cases except in those who showed obvious hematologic relapse.

*C. Neurologic Status of Patients.*—Neurologic relapse occurred in three of these forty-one patients. The most striking of these has been reported in detail in a previous publication.<sup>7</sup> The patient, who had Addisonian pernicious anemia, was seen in relapse (Case 41, Table IV). Folic acid therapy was begun in daily oral dosage of 10 milligrams. This was followed by a reticulocyte response of 40 per cent (calculated theoretic maximum, 22.3 per cent). There was an excellent rise in erythrocytes and hemoglobin, with complete disappearance of macrocytosis. Eighty-three days after beginning treatment there was a sudden and rapidly progressive onset of paresthesias with rapid loss of deep reflexes and vibratory sensation. At the time of onset of the neurologic relapse, the

blood findings were: erythrocytes, 5,090,000 per cubic millimeter; hemoglobin, 15.9 Gm. per 100 ml.; hematocrit, 48; mean corpuscular volume, 94.5 cubic microns. Vigorous liver extract therapy for twenty-seven days was necessary to produce the first evidence of arrest of the neurologic lesions. Since then the patient has made gradual and steady improvement until there is only a small amount of residual numbness of the fingers and toes. Deep reflexes are still absent and vibratory sensation has not improved. This is in keeping with the concept that degenerative lesions in the spinal cord are not reversible,<sup>12</sup> whereas paresthesias and peripheral neuritis are responsive to therapy.

This patient ate an inadequate diet throughout. At the time of institution of therapy he had only four teeth and this, combined with the naturally poor appetite seen in patients with untreated pernicious anemia, reduced his food intake. Shortly after remission was induced with folic acid he had the four remaining teeth extracted and the resulting sore mouth and complete absence of teeth limited his food intake to liquids for about three weeks. It should be emphasized that after hematologic remission was induced with folic acid the patient had a desire to eat but was restricted from doing so by the absence of teeth.

Another patient (Case 31, Table III) was a 51-year-old white woman with addisonian pernicious anemia who had developed severe sensitivity to substances in liver extract. After an unsuccessful trial of desensitization, she was placed on oral therapy with Extralin B (Lilly), 9 to 15 capsules daily. This dosage did not maintain the blood at satisfactory levels, and liver extract sensitivity was manifested by a continuous mild skin eruption. She was placed on oral folic acid therapy of 40 mg. daily, reduced gradually to 10 mg. daily. There was a marked improvement in the blood with an associated improvement in sense of well-being. The skin eruption completely disappeared. After a year, however, she developed soreness of the tongue and mouth, without objective changes, and increasing numbness of the feet and hands, the latter especially noticeable since the patient was a seamstress. Therapy with Extralin B was reinstituted at the rate of 6 capsules daily, and folic acid, 40 mg. daily, was given simultaneously. Over a period of weeks the soreness of the mouth disappeared and the numbness of the extremities improved. The skin eruption promptly returned. The patient also probably had inadequate food intake. Her appetite was never robust. She was an extremely "nervous" individual and when emotionally disturbed, which was frequently, she responded with nausea and vomiting so that she would eat little or nothing for two or three days at a time. Repeated x-ray examinations failed to reveal any organic disease of the gastrointestinal tract.

The third patient showing neurologic relapse was a 77-year-old white woman who had been maintained on weekly or biweekly injections of liver extract for many years with satisfactory blood values (Case 34, Table III). This patient also had a poor appetite, with considerable "indigestion," so that she voluntarily limited food intake with respect to both quantity and quality. She was changed to folic acid therapy, 45 mg. intramuscularly every two weeks. After only one month she developed slight but definite increase in numbness of the hands, soreness of the tongue, weight loss, and further decrease in appetite and



increase in "indigestion." She was again placed on intramuscular liver extract therapy (purified liver extract, 15 units per milliliter, Lilly) at weekly intervals and was given a hydrochloric acid-yielding tablet (Acidulin, Lilly) with meals. In a period of days the soreness of the tongue and numbness of the extremities improved and eventually disappeared. Her appetite increased to a degree which astonished her family and she became free of indigestion. Some credit for this improvement, at least as concerns the indigestion, should probably be given to the hydrochloric acid since she is in a better state now than when on liver extract therapy previously, without acid.

All three of the patients who developed evidence of neurologic relapse probably ate inadequately. Whether this contributed to the relapse cannot be stated, but it is not unlikely that inadequate diets may contain an insufficient amount of some substance, other than folic acid, which is able to prevent the onset of neurologic relapse.

*D. Patients Sensitive to Liver Extracts.*—Folic acid was given to two patients who, because of sensitivity to liver extract, had been maintained on oral medication with liver-stomach (Extralin, Lilly). One of these patients (Case 31) was referred to previously. The other (Case 32), a 77-year-old white woman with addisonian pernicious anemia, also had marked sensitivity to injections of liver extract and was subject to severe bronchial asthma. She had been taking Extralin (Lilly), 9 capsules daily, for several years. On this therapy the erythrocyte and hemoglobin levels (Table III) were not optimal. After institution of folic acid therapy there was considerable rise in the blood values. The patient experienced no subjective improvement, however.

Attempts at desensitization of these two patients were made without success. It has been our experience in general that liver extract sensitivity is apparently an organ sensitivity rather than a species sensitivity, as has been suggested.<sup>13</sup> It is also not uncommon, in our experience, to find that oral therapy with liver or stomach preparations may fail to maintain adequate blood levels. As demonstrated by the two patients cited, folic acid may well prove to be a valuable form of therapy in such liver extract-sensitive patients. However, in the presence of neurologic manifestations, additional oral liver therapy would be indicated.

### III. DISCUSSION

Discussion has begun to appear concerning the relative ability of folic acid and liver extract to produce and maintain hematologic remission. One author, previously cited,<sup>6</sup> believed that his data indicated that folic acid, unlike liver extract, rarely produced a maximal response in reticulocyte and erythrocyte levels, although he believed a combination of folic acid and liver extract was probably superior to liver extract alone. Another publication,<sup>14</sup> in which the effects of liver extract and folic acid were compared, suggested that the response to folic acid may have been somewhat slower than with liver extract but eventually as good. These authors believed, however, that some patients maintained on folic acid required increasing doses as time elapsed.

In the series of cases studied here, there was no indication that folic acid was unable to produce a maximal reticulocyte response when given in doses of

10 mg. or more daily to patients in relapse, with the one exception cited previously. One patient in particular (Case 41), who later showed neurologic relapse, had a reticulocyte response of 40 per cent when the expected theoretic maximal as calculated for liver extract was 22.3 per cent. This response was followed by an excellent increase in red count and hemoglobin to high normal levels and by disappearance of macrocytosis in a period of sixty-one days.

Similarly, during the period of observation of this group of cases, folic acid showed no inability to maintain the erythrocyte count at high levels when given in sufficient amount. "Sufficient amount" is dictated more by interval than by size of dose. Intermittent medication at intervals of longer than two weeks did not consistently prevent hematologic relapse in these patients. Daily oral administration of 10 mg. was effective in preventing hematologic relapse during the period of observation, which indicates that this method of treatment is the one of choice.

No toxic or allergic manifestations to folic acid have been observed to date except after rapid intravenous administration of large amounts (150 mg. in 7.5 ml. of solution). Several patients so treated responded with vertigo, syncope, and marked drop in blood pressure. Recovery was always complete within two or three minutes, except for a headache, which persisted up to twenty-four hours. Since intravenous medication has no advantage over oral or intramuscular use and since it has the possible disadvantage of even more rapid urinary excretion, there is no indication for intravenous administration. Whether these reactions were due to high concentration of folic acid in the blood or to impurities present in the solution was not determined.

#### IV. CONCLUSIONS

1. Forty-one patients with pernicious or allied macrocytic anemia were treated with folic acid for periods up to one year.

2. Of these, twenty-six showed no hematologic relapse, nine had a decrease in erythrocyte count, and six showed an improvement when the results were compared with those obtained during previous therapy with liver extract.

3. All nine who developed hematologic relapse were given intermittent intramuscular injection of either 50 or 150 mg. of folic acid at intervals of three or more weeks.

4. Of the six showing hematologic improvement, as compared with previous liver extract therapy, three were given daily oral therapy, two were given intramuscular injections at intervals of two weeks, and only one was given intramuscular therapy at an interval longer than two weeks.

5. Intermittent intramuscular therapy with folic acid at intervals greater than two weeks cannot be relied upon to maintain hematologic remission with individual doses as great as 150 mg. Daily oral medication of 10 mg. did not allow hematologic relapse in any patient observed.

6. Six patients given folic acid while in hematologic relapse had a satisfactory reticulocyte response and increase in erythrocytes and hemoglobin.

7. One patient was encountered who showed very minimal hematologic response when given daily intramuscular injections of 12 mg. of folic acid.

A prompt and theoretically maximal response ensued when liver extract therapy was instituted.

8. Three of the forty-one patients developed neurologic relapse when the erythrocytes and hemoglobin were at normal levels on folic acid therapy. These patients responded to liver extract therapy.

9. In spite of its inability to prevent neurologic relapse, folic acid has a place in the therapy of macrocytic anemia, particularly in those patients who are sensitive to extracts of liver. In such instances, its combination with oral liver extract preparations might be desirable.

10. Toxic or allergic manifestations to folic acid have not been observed except for mild nitritoid reactions following rapid intravenous administration.

11. Because of neurologic relapses occurring in patients treated with folic acid, this drug cannot be considered a complete substitute for liver extract, and liver extract, or liver extract combined with folic acid, must remain the treatment of choice for pernicious anemia.

#### REFERENCES

1. Spies, T. D., Vilter, C. F., and Koch, M. B.: Observations of the Anti-Anemic Properties of Synthetic Folic Acid, *South. M. J.* 38: 707, 1945.
2. Moore, C. V., Bierbaum, O. S., Welch, A. D., and Wright, L. D.: The Activity of Synthetic *Lactobacillus casei* Factor ("Folic Acid") as an Anti-Pernicious Anemia Substance. I. Observations on Four Patients: Two With Addisonian Pernicious Anemia, One With Nontropical Sprue, and One With Pernicious Anemia of Pregnancy, *J. LAB. & CLIN. MED.* 30: 1056, 1945.
3. Spies, T. D.: Effect of Folic Acid on Persons With Macrocytic Anemia in Relapse, *J. A. M. A.* 130: 474, 1946.
4. Darby, W. J., Jones, E., and Johnson, H. C.: Effect of Synthetic *Lactobacillus casei* Factor in Treatment of Sprue, *J. A. M. A.* 130: 780, 1946.
5. Zuelzer, W. W., and Ogden, F. N.: Folic Acid Therapy in Macrocytic Anemias of Infancy, *Proc. Soc. Exper. Biol. & Med.* 61: 176, 1946.
6. Meyer, L. M.: Folic Acid in the Treatment of Pernicious Anemia, *Blood*, *J. Hemat.* 2: 50, 1947.
7. Heinle, R. W., and Welch, A. D.: Folic Acid in Pernicious Anemia. Failure to Prevent Neurologic Relapse, *J. A. M. A.* 133: 739, 1947.
8. Vilter, C. F., Vilter, R. W., and Spies, T. D.: The Treatment of Pernicious Anemia and Related Anemias With Synthetic Folic Acid. I. Observations on the Maintenance of a Normal Hematologic Status and on the Occurrence of Combined System Disease at the End of One Year, *J. CLIN. & LAB. MED.* 32: 262, 1947.
9. Steinkamp, R., Shukers, C. F., Totter, J. R., and Day, P. L.: Urinary Excretion of Orally Administered Pteroylglutamic Acid, *Proc. Soc. Exper. Biol. & Med.* 63: 556, 1946.
10. Welch, A. D., and Heinle, R. W.: Unpublished data.
11. Darby, W. J.: *Vitamins and Hormones*, Academic Press, Inc., New York, vol. 5. In press.
12. Rundles, R. W.: Prognosis in the Neurologic Manifestations of Pernicious Anemia, *Blood*, *J. Hemat.* 1: 209, 1946.
13. Editorial: Folic Acid, *New England J. Med.* 236: 219, 1947.
14. Frommeyer, W. B., Jr., and Spies, T. D.: Relative Clinical and Hematologic Effects of Concentrated Liver Extract, Synthetic Folic Acid and Synthetic 5-Methyl Uracil in the Treatment of Macrocytic Anemias in Relapse, *Am. J. M. Sc.* 213: 135, 1947.

# PERITONEAL IRRIGATION IN THE TREATMENT OF RENAL FAILURE DUE TO TRANSFUSION REACTION

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THE recent report of successful peritoneal irrigation in a patient suffering from renal failure following the administration of sulfathiazole<sup>1</sup> prompted us to use a similar method in the treatment of a man who developed severe renal damage following a transfusion reaction. During the course of peritoneal irrigation, the patient developed generalized edema. Death occurred on the eighth day following transfusion. Because the Tyrode's solution used for irrigation contained a higher concentration of sodium chloride than did plasma, it seems possible that the generalized edema was due to salt retention from the irrigating fluid. Because of the appearance of edema during the course of therapy, this patient is of particular interest.

## CASE REPORT

The patient was a white man, 29 years of age, with tuberculosis of the eighth and ninth thoracic vertebrae and a large paravertebral abscess. He was afebrile and the general condition was good. An intravenous pyelogram and repeated urinalyses were normal.

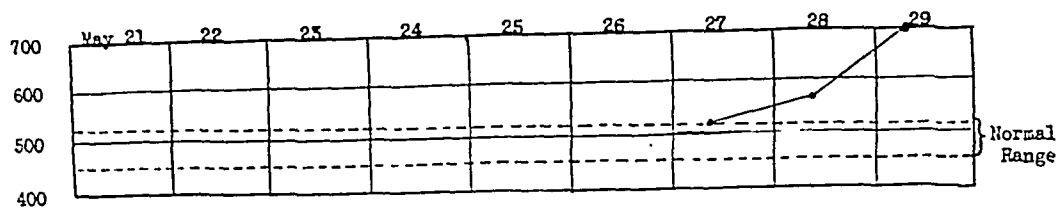
On May 21, 1946, spinal fusion was performed, and during operation a blood transfusion of 500 ml. was given. Cyanosis, unrelieved by oxygen administration, was observed by the anesthetist during and after the transfusion. The total urine output for the following days is shown in Table I.

The oliguria was first noted twenty hours after transfusion and 2,000 ml. of 5 per cent glucose in distilled water and 1,000 ml. of  $\frac{1}{6}$  M sodium R lactate were given intravenously. A specimen of venous blood showed no gross evidence of hemolysis and the icterus index was 20. On the supposition that the renal suppression might be reflex in origin, efforts were made to stimulate renal excretion by the intravenous administration of 100 ml. of 25 per cent glucose and 40 ml. of aminophyllin (4.0 Gm.). A high spinal anesthetic and continuous gastric suction were also used. Two days after transfusion the incompatibility of the transfused blood was established. The donor was found to be Group A and the recipient Group O. The patient's blood nonprotein nitrogen gradually became elevated (Fig. 1, Section B) and attained a level of 126 mg. per 100 ml. on May 25 (four days after transfusion). The patient was drowsy, restless, and irritable and the abdomen was distended. Peritoneal irrigation with Tyrode's solution was instituted at 3:00 P.M. on May 25. The following formula was used:

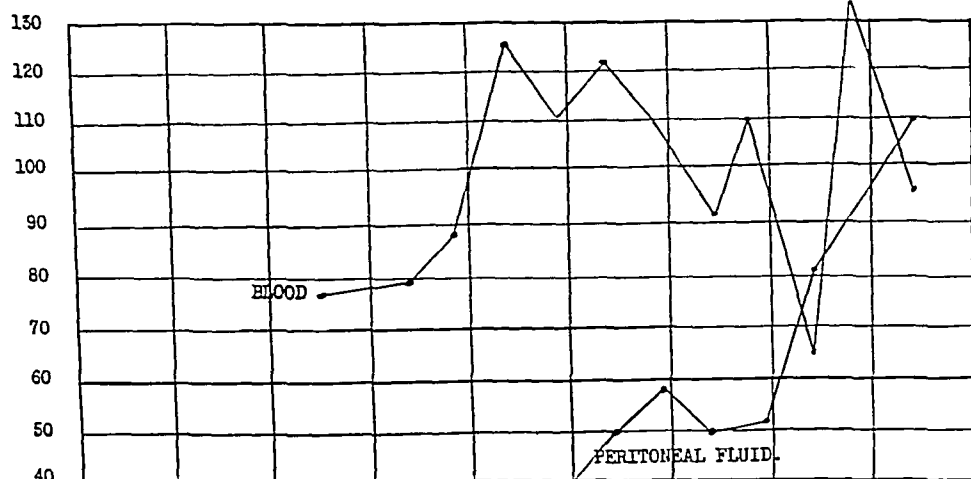
	MG. %
NaCl	800
KCl	20
CaCl <sub>2</sub>	20
MgCl <sub>2</sub>	10
NaH <sub>2</sub> PO <sub>4</sub>	5
Glucose	100
NaHCO <sub>3</sub>	100

All the ingredients except the sodium bicarbonate were dissolved and autoclaved in 2-liter bottles. The sodium bicarbonate was dissolved and autoclaved separately. The fluid

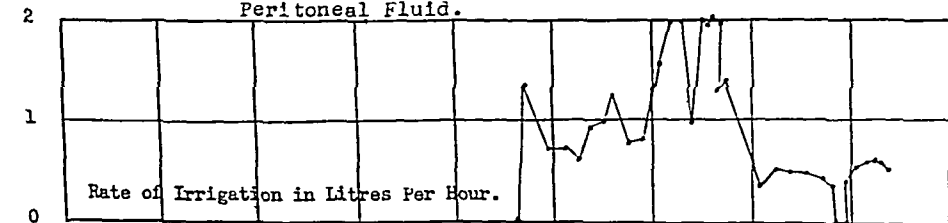
Received for publication, Dec. 26, 1946.



SECTION A. Blood NaCl in Mg. per 100 c.c.



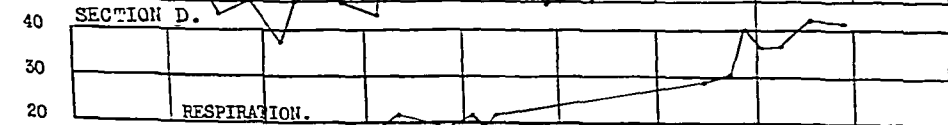
SECTION B. N.P.N. in Mg. per 100 c.c. of Blood and Peritoneal Fluid.



SECTION C.



SECTION D.



SECTION E.

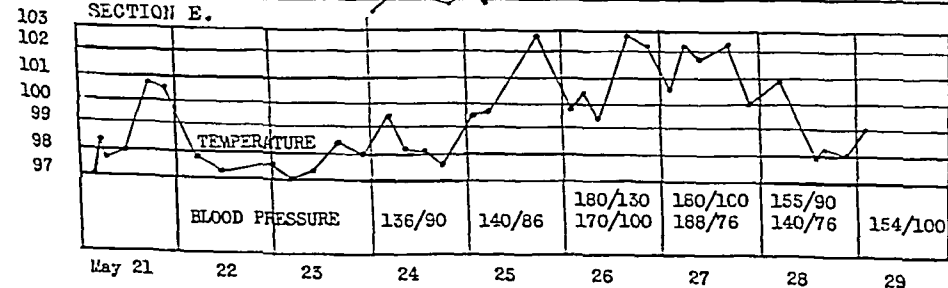


Fig. 1.

TABLE I

DATE	TOTAL URINE VOLUME (ML.)	URINALYSIS
5/22/46	30	
5/23/46	60	Alk.; alb, 3+; R.B.C., 2+; W.B.C., 3+
5/24/46	60	Alk.; alb, 2+; R.B.C., 1+; W.B.C., 1+
5/25/46	60	Alb.; alb, 2+; R.B.C., 1+; W.B.C., 1+
5/26/46	55	
5/27/46	Nil	
5/28/46	30	
5/29/46	Nil	

was warmed in a water bath at 38° C. before being used, at which time the sodium bicarbonate was added together with the following: heparin, 5 mg. per liter; penicillin, 7,500 units per liter; and streptomycin,\* 10,000 units per liter. The fluid was admitted to the peritoneal cavity through a mushroom catheter to the left of and above the umbilicus and removed by an abdominal sump suction apparatus directed into the pelvis through an incision in the right lower quadrant. Continuous suction was maintained by a Stedman pump and the suction device was adequate to keep up with any given rate of intake. The greatest rate of flow was 2 liters per hour, the average rate being 1 liter per hour (Fig. 1, Section C). The outcoming fluid was slightly yellowish-green in color and definitely turbid. Peritonitis was guarded against by sterile technique, penicillin dressings around the peritoneal tubes, intramuscular penicillin, and penicillin and streptomycin in the irrigating fluid. The outlet fluid was sterile at all times, and at autopsy there was no evidence of wound infection or peritonitis. The irrigation was maintained continuously except for a lapse of a few minutes on May 27 and for a period of three hours on May 28.

On the morning following the commencement of the irrigation the patient showed considerable subjective improvement. Slight subcutaneous edema of the buttocks and ankles was noted at this time, but the chest was apparently clear. From this time on, the picture was one of slowly progressive generalized edema. Moist râles were heard at the bases of both lungs and on May 27 the patient became dyspneic. On this date a venesection with removal of 500 ml. of blood was performed and 150 ml. of twice concentrated plasma were given without apparent effect. The edema progressed and the patient died on May 29, seven days after the administration of the incompatible blood and four days after the institution of peritoneal irrigation.

In addition to the treatment described, the patient received vitamin C and vitamin B complex. Continuous oxygen by B L B mask was given from May 27, and frothy tracheal fluid was repeatedly aspirated. Intravenous fluids were administered as shown in Table II.

During the course of observation the gastric contents were removed through a Levine tube, but this was tolerated only about one-half of the time. While the tube was out the

TABLE II

DATE	FLUID	AMOUNT (ML.)
5/22/46	5% glucose in distilled water	2,000
	$\frac{1}{2}$ M sodium R lactate	1,000
5/23/46	5% glucose in distilled water	1,500
	25% glucose in distilled water	100
5/24/46	10% glucose in distilled water	500
5/25/46	10% glucose in distilled water	500
	10% glucose in normal saline	500
5/26/46	Plasma	500
	10% glucose in distilled water	150
	Twice concentrated plasma	150
5/28/46	Nil	
5/29/46	Nil	

\*The streptomycin was supplied through the courtesy of Dr. L. E. Ranta, Western Division of Connaught Laboratories, Vancouver, B. C.

patient drank small quantities of fluid and vomited intermittently. The blood carbon dioxide combining power of the patient varied between 40 and 66 volumes per cent during the period of observation.

*Autopsy Findings.*\*—Autopsy revealed the following: (1) acute nephrosis (transfusion kidneys), (2) pulmonary edema (marked), (3) generalized edema, (4) cardiac dilatation, (5) miliary tuberculosis of lungs, liver, and spleen, (6) vertebral tuberculosis and para-vertebral abscess, (7) operation (spinal fusion).

The cause of death was pulmonary edema. The abdominal cavity contained about 100 ml. of thin yellowish fluid. There were no fibrinous adhesions over the intestines and the peritoneal surfaces were smooth and glistening. The two abdominal stab wounds were clean and in no place was there evidence of pressure on the viscera by the drainage tubes. The liver weighed 2,200 grams and was large and dark red in color. Its cut surface showed normal architecture. The spleen weighed 450 grams and had old fibrous adhesions at its upper pole. The right kidney weighed 280 grams and the left, 260 grams. They were large but not tense. The cut surfaces revealed pale yellowish-pink cortices measuring 0.6 to 0.7 cm. in thickness. The pyramids were a deeper pink. No petechial hemorrhages were present. The renal pelvis were of normal size and their lining membranes were pale.

The most striking microscopic change in the kidneys was dilatation of all convoluted and straight tubules, with flattening of their epithelium, and the presence of pink-staining fibrillar material in their lumina. Some tubules contained solid, brownish-red amorphous material in this situation. The interstitial tissue in scattered regions was a little loose and edematous and in a few scattered places there was some infiltration with lymphocytes. There was no evidence of renal tuberculosis.

#### COMMENT

That the irrigation was effective in reducing the blood nonprotein nitrogen is suggested by the large amount of nonprotein nitrogen removed in the drainage fluid (Fig. 1, Section B) which, on May 29, reached a level, in a twelve-hour aliquot specimen, of 110 mg. per 100 ml., and by the decline in blood nonprotein nitrogen following the institution of the irrigation. The blood nonprotein nitrogen reached its lowest level following a period of irrigation at a rate of 2 liters per hour, only to rise again during the time when the irrigation was slowed or temporarily stopped (compare Section B and C in Fig. 1). It seems reasonable to assume that during this four-day period the nonprotein nitrogen would otherwise have risen steadily.

The outstanding feature of the case was the progressive edema and there seems to be no doubt that this was the main factor in causing death. In considering the cause of the edema the following points are important.

*Fluid Intake.*—The intravenous fluid intake was not excessive (Table II). During eight days of observation the patient received only 6,900 ml., an average of only 860 ml. daily. The oral intake could not be measured accurately enough for record purposes. The patient frequently drank small quantities of water, but also vomited frequently and had a stomach tube (with suction) in place a considerable part of the time. It seems likely that any excess of oral intake over oral output was very small indeed and it may be that the balance was negative.

*Fluid Loss.*—The respiratory rate was consistently above normal (Fig. 1, Section E) and the patient perspired profusely, causing considerable fluid loss.

\*Autopsy performed by Dr. H. K. Fidler, Shaughnessy Hospital, Vancouver, B. C.

The urine output was only 295 ml. in eight days. During this period one enema was given, with good return, and there were three spontaneous and involuntary bowel movements. There was no diarrhea and the stool appeared normal.

*Protein Balance.*—Protein loss was considerable (Table III) but the effect on the plasma protein was not great.

TABLE III

DATE	PROTEIN CONTENT OF OUTFLOW FLUID	PLASMA PROTEIN
	(GM. PER 100 ML.)	(GM. PER 100 ML.)
5/25/46	0.38	6.2
5/26/46	0.10	5.12
5/27/46	0.06	5.4

*Chloride Balance.*—On one occasion the patient was given 4.5 Gm. sodium chloride in the form of normal saline. The oral intake was negligible. In spite of this the blood chlorides (expressed as NaCl) showed a progressive rise from 520 to 690 mg. per 100 ml. (Fig. 1, Section A).

In reviewing the foregoing data, it would appear that the edema was not the result of too great a fluid or chloride intake by the intravenous or oral routes. Another possible source of edema-producing fluid was that which flowed through the peritoneal cavity. Its electrolyte content was considerably higher than that of normal blood plasma, and it seems likely that while the nonprotein nitrogenous substances were passing in one direction, electrolytes accompanied by water were passing in the opposite direction and producing hydremia and hyperchloremia.

We can find no other explanation for the rising blood chlorides. In the case reported by Frank, Seligman, and Fine<sup>1</sup> the blood chloride remained high (660 mg. per 100 ml.) and it is noteworthy that pulmonary edema developed in their patient on the third day of irrigation. It may be that if the kidneys had not started to function on the following day, the edema might have progressed. This raises the question as to whether or not Tyrode's solution is ideal.

The normal range of serum chloride (expressed as sodium chloride) is between 570 and 620 mg. per 100 milliliters. The Tyrode's solution used in irrigation contained 800 mg. of sodium chloride per 100 milliliter. Since sodium chloride forms a crystalloidal solution, it would pass readily through the peritoneal membrane until the concentration in the serum approximated the concentration in the peritoneal irrigating fluid.

The assumption that the increased chloride was responsible for the tissue edema is supported by the following passage from Stitt and co-workers:<sup>2</sup> "One of the major functions of sodium chloride is to maintain the normal osmotic pressure in the body fluids. Some unknown mechanism, in which the kidney must play the major role, keeps the electrolyte content of the plasma nearly constant. . . . If chloride is ingested faster than it can be excreted, it does not accumulate in the plasma but passes (as sodium chloride and water) into the intercellular tissue spaces and produces edema."



In view of the foregoing, it would appear logical to modify the formula of the peritoneal irrigating fluid for future cases so that it contains 570 mg. of sodium chloride rather than 800 mg. per 100 milliliters.

#### CONCLUSION

In a case of renal failure caused by a blood transfusion reaction peritoneal irrigation with Tyrode's solution produced an apparent beneficial effect on the uremic manifestations, but the patient died because of generalized edema.

The suggestion is made that a modification of Tyrode's solution (reduction chloride content) might prevent such edema.

#### REFERENCES

1. Frank, H. A., Seligman, A. M., and Fine, J.: Treatment of Uremia After Acute Renal Failure by Peritoneal Irrigation, *J. A. M. A.* 130: 703, 1946.
2. Stitt, E. R., Clough, P. W., and Clough, M. C.: *Practical Bacteriology, Hematology and Parasitology*, ed. 9, Philadelphia, 1938, The Blakiston Co., p. 668.

# PERITONEAL IRRIGATION FOR ACUTE RENAL DAMAGE FOLLOWING INCOMPATIBLE BLOOD TRANSFUSION: A DISCUSSION BASED ON THREE CASES

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PERITONEAL irrigation as a procedure for clearing waste products from the body has been attempted on several occasions in the past.<sup>1-4</sup> Recently this procedure has been rejuvenated as a result of improved techniques and results presented by Fine, Seligman and Frank.<sup>5-7</sup> Other reports<sup>8-10</sup> have resulted from the stimulus initiated by these workers.

We have conducted this procedure of peritoneal lavage on three cases of renal insufficiency resulting from incompatible blood transfusion.\* Since experience with the first case, there has also been the opportunity to observe the results of different types of management (mentioned later) on additional identical cases and other related types of acute renal damage. The latter will be presented in a review of all known hemolytic transfusion reactions from this hospital since the inception of the blood bank in 1939.<sup>11</sup> An appraisal of the peritoneal irrigation technique resulting from this experience seems appropriate.

## TECHNIQUE OF PERITONEAL IRRIGATION

*Introduction of Inlet Catheter and Outlet Drain.*—The arrangement for irrigating the peritoneum has in each case identical in its main features to that presented by Fine, Seligman, and Frank.<sup>5,7</sup> Under local anesthesia (novocain) and through a low lateral McBurney incision a "sump" drain<sup>7,12</sup> (Fig. 1) was inserted into the right lower quadrant and placed in the right paracolic gutter adjacent to the cecum. The incision was sutured snugly about the upper collar of the "sump" drain. Through a minimal skin incision a thoracentesis trocar was introduced through the abdominal wall of the left upper quadrant two inches below the costal margin. A No. 18 French catheter was introduced well into the peritoneal cavity and the trocar was removed. The skin edges were sutured snugly about the catheter. The entire procedure, conducted in the operating room, was completed within thirty to forty-five minutes.

*Irrigating Equipment.*—The fluid was allowed to enter through the catheter by gravity and was drained out through the "sump" drain into a 20-liter bottle to which slight negative pressure was applied by a Wangenstein suction or a small electric (aspiration) suction pump. On two cases a bacterial Mandler filter was interposed between the entering fluid reservoir and the intake catheter. No additional pressure was necessary for filtration at the desired rate of flow.

*Irrigating Solutions.*—Mammalian Tyrode's solution as designated by Seligman and co-workers<sup>6</sup> was most frequently used. On two occasions 5 per cent dextrose in distilled water was deliberately used (see Cases 1 and 2). At other times and for short intervals a sixth-molar sodium lactate and three chlorides (Ringer's) solution was used.

The solutions were prepared in two different manners. In the first case separately sterilized solutions combined prior to utilization were used. In two cases freshly distilled

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\*Two cases in our hospital, one case elsewhere.

water was collected in 20-liter bottles and the previously weighed solid ingredients were added just before use. The latter was much more practical in view of the interposed Mandler bacterial filter.

To each liter of irrigating fluid were added 25,000 units of penicillin and 1 mg. of heparin. During the irrigation, penicillin also was given intramuscularly (usually 20,000 units every three hours). Two cases received streptomycin by peritoneum and muscle during the latter phases of the irrigation.

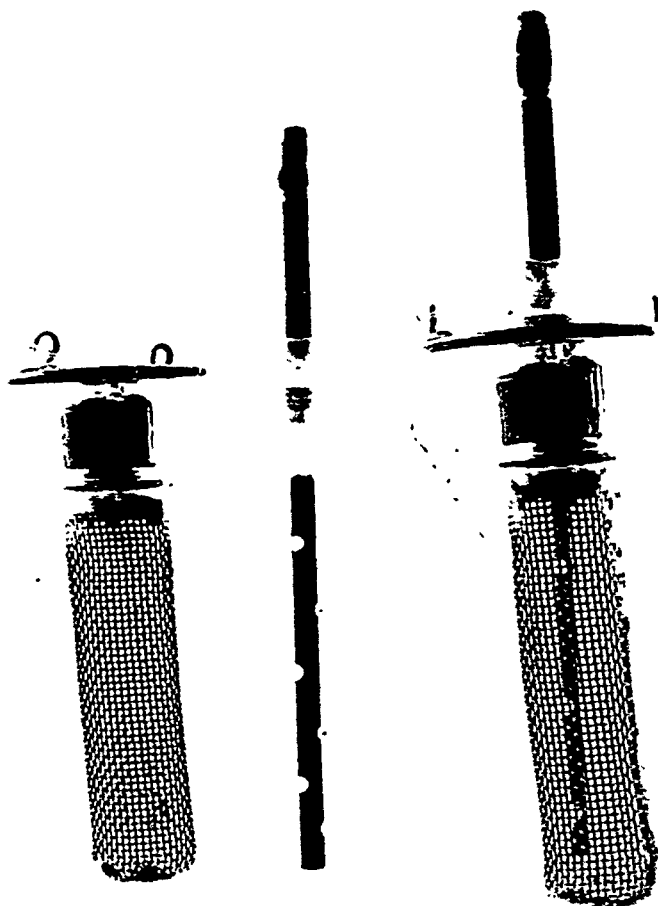


Fig. 1.—The type of "sump" drain used is represented. The abdominal wall was made to fit snugly about the collar and the wire-mesh portion was placed in the right paracolic gutter.

Once started, the irrigation was continuous in two cases and interrupted by a twelve-hour period in one case. The rate of flow was near 1,000 ml. per hour.

Attempts were made to give a high caloric formula by mouth but were not completely successful. Glucose and amino acid were given, but usually not enough for basal dietary demands. Vitamin B fractions, vitamin C, and calcium gluconate were given parenterally. Multiple transfusions were given for anemia.

#### CASE REPORTS

Whenever possible, a check on the fluid intake by all portals and on the urine output was maintained. A slight but variable leakage always occurred about the "sump" drain, making the measurement of outflow fluid and total urea recovered

somewhat lower than the true value. The urine urea and chloride content were checked daily in two cases. The blood urea,<sup>13</sup> chloride<sup>14</sup> and hemoglobin concentration,<sup>15</sup> and the CO<sub>2</sub> combining power of the plasma<sup>16</sup> were checked frequently. Several serum sodium and potassium concentration determinations<sup>17</sup> were made.

CASE 1.—This case has been discussed elsewhere<sup>10</sup> and only essential points will be mentioned. A 36-year-old white woman was given a transfusion for a moderate blood loss anemia. Through a clerical error type A<sub>1</sub>, D-positive\* blood was given while the patient was of type O, D positive. A severe reaction developed after 175 ml. were given and the transfusion was discontinued.

There was prominent oliguria for twelve days but during the thirteenth, fourteenth, fifteenth, and sixteenth days a steadily increasing urinary output occurred (see Table I). During these sixteen days the fluid intake amounted to some 25,750 ml. (17,000 ml. by vein with 70 Gm. NaCl). There was persistent vomiting. The patient's condition deteriorated markedly. There was azotemia (blood urea, 310 mg. per cent), hypochloremia (blood chlorides, 360 mg. per cent), and acidosis (CO<sub>2</sub> combining power, 40.5 volumes per cent). The following signs developed: gallop rhythm, apical systolic murmur, pericardial friction rub, generalized and subcutaneous edema, clonic muscular spasms, blood pressure, 160/110 mm. Hg, and mental confusion.

Peritoneal irrigation over a period of 5.5 days (sixteenth to twenty-second day) was responsible for the clearance of over 76.84 Gm. of urea from the body.

Despite some apparent improvement during the first three days of irrigation (greater mental clearness, diminished muscular jerks, etc.) the patient became more edematous and the cardiac embarrassment seemed more pronounced.† During this time there was a distinct elevation in the blood chloride and serum sodium concentration but the serum potassium concentration remained normal. The hemoglobin concentration of peripheral blood remained at the same level despite prior transfusions of blood. The acidosis became more pronounced. The blood urea concentration was markedly lowered, a lowering not in keeping with the quantity of recovered urea and, therefore, considered as partly a dilution phenomenon. Vomiting and retching continued. It became quite obvious that notwithstanding an already existing extracellular water-salt overload, water and salts continued to be absorbed from the peritoneal surfaces, thus accentuating an already existing complication.

Dramatic beneficial effects in this case were associated with the washing of the peritoneal surfaces with 5 per cent dextrose in distilled water at this time for a continuous twenty-four-hour period. The recovered solution had a composition of 2.5 per cent dextrose and 0.42 per cent NaCl. By this means alone some 60 to 70 Gm. of NaCl were removed from the body. This correction of the salt overload was concomitantly associated with a copious diuresis. A subsequent transient oliguria developed (partly related to water deficit) which, when corrected, was followed by recovery.

Peritonitis due to *Proteus morgani* was treated with streptomycin. It is regrettable to report the development of vestibular damage which did not become evident until after the patient was discharged from the hospital. Otherwise the patient has recovered.

*Comment.*—Certain distinct complications occurred during the irrigation period: (1) an accentuation of the extracellular overhydration (pulmonary and subcutaneous edema), (2) an accentuation of the extracellular salt overload, (3) the development of severe acidosis, and (4) peritonitis without gaseous distention. Retching and vomiting continued until the catheter and drain were removed.

In the light of subsequent experience gained with these and related cases since the time of the report of this first case, additional interpretations seem

\*The antigen D of the Fisher-Race Rh concept.<sup>18, 19</sup>

†A digitalis effect disappeared when the drug was discontinued.

TABLE 1 (CASE 1)

DAY	BLOOD (MG. %)		CO <sub>2</sub> C.P. (VOL. %)	BLOOD C.I. (MEQ./L.)	SIRUM NR (MEQ./L.)	SIRUM K (MEQ./L.)	PLASMA PROT. CONC. (GM. %)	Hb. (GM. %)	DAYS	FLUID INTAKE (APP.) (ML.)	URINE VOL. (ML.)	URINE UREA (GM.)	URINE CL (NaCl) (GM.)	PERIT. FLUID VOL. (ML.)	PERIT. UREA (GM.)	EMESIS VOL. (APP.) (ML.)	B.P. (MM. HG)
	UREA	N															
2	63	135		86.2			5.7	10.8	1-4	5,500	335					1,420	120/70
7-8	107	229	58	67.6				9.2	5-8	7,600	575					2,120	134/80
12	115	246	48	64.2				10.4	9-12	6,000	1,935					2,620	140/80
16	150	310	49	60.8					13-16	6,650	6,600					1,300	160/110
17	88.2	189	46					10.2	17	1,925	2,300	5.2	3.3	11,900	15.9	1,920	150/100
18	100	214		93			6.7		18	2,100	2,130	12.9	8.5	16,000	16.48	2,110	150/100
19	83	178	31	105	150	4.84		10.8	19	2,210	1,725	11.4	6.8	17,800	13.6	900	130/85
20	79	169	36	94.3					20	2,125	4,375	15.0	8.2	8,400	5.9	1,310	125/80
21	56	120	30	71					21	2,475	2,475	11.3	1.5	19,865	11.6	80	100/70
22	50	107	30	74.3				15.8	22	2,840	1,750	10.9	1.3	18,840	13.36	900	120/82
25	43	92	56	74.3				10.4	23-28	25,670	20,565	156.7	354			2,750	120/85
29	37	79	54	67.6					29-34	15,600	17,770	132.8	24.3			600	
35	20	43		72.7	142	4.1	6.6	15.0	35-40	19,700	16,585	120.3	35.2				
46	15	32	58	81			7.1		41-46	17,600	21,820	103.8	42.5				

Peritoneal irrigation was conducted in the interval between the seventeenth to twenty-second day. Notice the recovery of substantial urea, but at the same time a marked decrease in the CO<sub>2</sub> combining power of plasma and a prominent increase in the blood chloride concentration. The chloride concentration was lowered after the use of the glucose solution as irrigating fluid, but the acidosis persisted. The average normal blood chloride concentration by the method used is 84.5 meq. per liter (or 400 me. per 100 ml. of NaCl). The fluid intake volume is not completely accurate since at times estimations of the oral intake were made. The tendency was for these figures to be conservative rather than excessive. The intravenous fluid intake was always accurately known. The urine chlorides are listed as grams of NaCl. This is purposely done in order to compare the figures with the grams of urea in the urine. The various abbreviations are self-explanatory.

justified. The usual time interval for kidney regeneration (structural and functional) had already elapsed by the time the irrigation was commenced. Extraneous factors such as the extracellular salt overload seemed to have interfering influences on renal function as reflected by the occurrence of diuresis and recovery when the salt-removing procedure was instigated. Such reasoning suggests that mostly extrarenal rather than truly renal causes were dealt with by the irrigation procedure in this case.

CASE 2.—This patient, a thirty-year-old white woman, had extensive tuberculosis of the right lung for which a thoracoplasty operation (first and second stage) was performed. She was well nourished.

Unmistakable hemolytic transfusion reaction occurred through no fault of anyone concerned. The patient's blood was typed on many occasions and each time was found to be of type A<sub>1</sub>, D positive. All donors giving blood to this patient were rechecked and each was found to be of type A<sub>1</sub>, D positive.\*

	ANTI-C	ANTI-D	ANTI-E	ANTI-C
Patient	+	+	+	+
Donor 1	+	+	—	+
Donor 2	+	+	—	+
Donor 3	—	+	+	+
Donor 4	+	+	—	+
Donor 5	+	+	—	+
Donor 6	+	+	—	—
Donor 7	+	+	—	+
Donor 8	+	+	+	+

Uneventful blood transfusions were given during the first phase thoracoplasty. During and after the second phase operation, two weeks later, transfusions were followed by hemoglobinuria, jaundice, and the typical type of renal insufficiency. Multiple attempts to demonstrate antibodies in the patient's serum (agglutinating, "blocking," and the "third order" types<sup>20</sup>) were fruitless. The subsequent autopsy demonstrated typical renal lesions of incompatible transfusion type (Figs. 2 and 3).

While under anesthesia (pentothal-cyclopropane-oxygen) during the second phase thoracoplasty the patient received 500 ml. of blood and 500 ml. of 5 per cent dextrose solution. For two hours following the operation the blood pressure remained near 80/60 mm. Hg. Following 500 ml. of dilute plasma the blood pressure became 104/70. At this time (three hours after operation) an additional 500 ml. of blood were given. Nausea and vomiting appeared but the blood pressure remained 114/80. Nine hours after the operation 200 ml. of very dark urine were voided.

Oozing of blood from the operative site began and the blood pressure dropped to 78/50 mm. Hg. After 500 ml. of dilute plasma the blood pressure was 105/70 (nineteen hours after the operation). Under cyclopropane anesthesia the wound was reopened and packed while the patient received 750 ml. of blood, 1,000 ml. of 5 per cent dextrose, and 500 ml. of dilute plasma (blood pressure, from 85/60 to 126/90). At this time 90 ml. of dark brown urine were voided and the patient was distinctly jaundiced (see Table II for icteric index).

The urine specimens during the second twenty-four hours (110 ml.) were likewise brown in color and contained much brownish granular pigment. Because of an alarming lowering of the hemoglobin concentration (5.6 Gm. per cent) the patient was given transfusions on the tenth, eleventh, and twelfth days amounting to 900 milliliters. Again the icteric index was elevated (additional hemolysis).

By summary then, this patient received 2,650 ml. of blood in a twelve-day period, much of which was rapidly hemolyzed through undemonstrated mechanisms.

It was decided to commence peritoneal irrigation as soon as renal insufficiency became definite and severe. We had in mind circumventing advanced renal insufficiency. The total urine volume for a little over three days was 300 to 400 ml., the blood urea concentration was 214 mg. per 100 ml., and, accordingly, the irrigation was initiated.

\*The patient's and donors' red cells gave the following results with anti-C, anti-D, anti-E, and anti-c sera of Race.

Peritoneal irrigation was conducted with 5 per cent dextrose in distilled water during the first seven hours as a means of checking the ability of this solution to wash out salts by this technique (refer to Case 1). During this time the solution entered as 5 per cent dextrose and was recovered with the following concentrations: dextrose, 3.9 per cent; chlorides, as NaCl 0.21 per cent; sodium, 91 mg. per 100 ml.; potassium, 8 mg. per 100 milliliters. Thus, in the 5,100 ml. actually recovered at this time the following bulk quantities were measured: 10.7 Gm. of NaCl, 4.64 Gm. of sodium, and 0.4 Gm. of potassium. Additional evidence was gained



Fig. 2.—The gross picture of a kidney from Case 2. The weight was twice normal and the cut surface bulged markedly. The medulla was dusky and striated due mainly to peritubular hyperemia. The cortex was light in color.

that this procedure can wash out substantial salt just as in the Darrow-Yannet experiment.<sup>21</sup> At the same time some 77 Gm. of dextrose were absorbed.

The irrigation was continued for seven and one-half days. Mammalian Tyrode's solution was used 75 per cent of the time; lactate-Ringer's solution, 14 per cent; and 5 per cent dextrose, 11 per cent. Table II contains the data on this case. The entire amount of urea measured in the outflow fluid was 115 grams. The azotemia abated considerably but again a severe acidosis developed, and some increase in the plasma chloride and sodium concentration occurred.

Most of the 4,025 ml. of fluid given on the first and second days consisted of blood and dilute plasma given to combat blood loss and hypotension. From the third to the eleventh day (irrigation period) the fluid intake, other than by peritoneum, amounted to 13,030 ml. and was given to equalize the insensible water loss and slight urinary output. On this regime

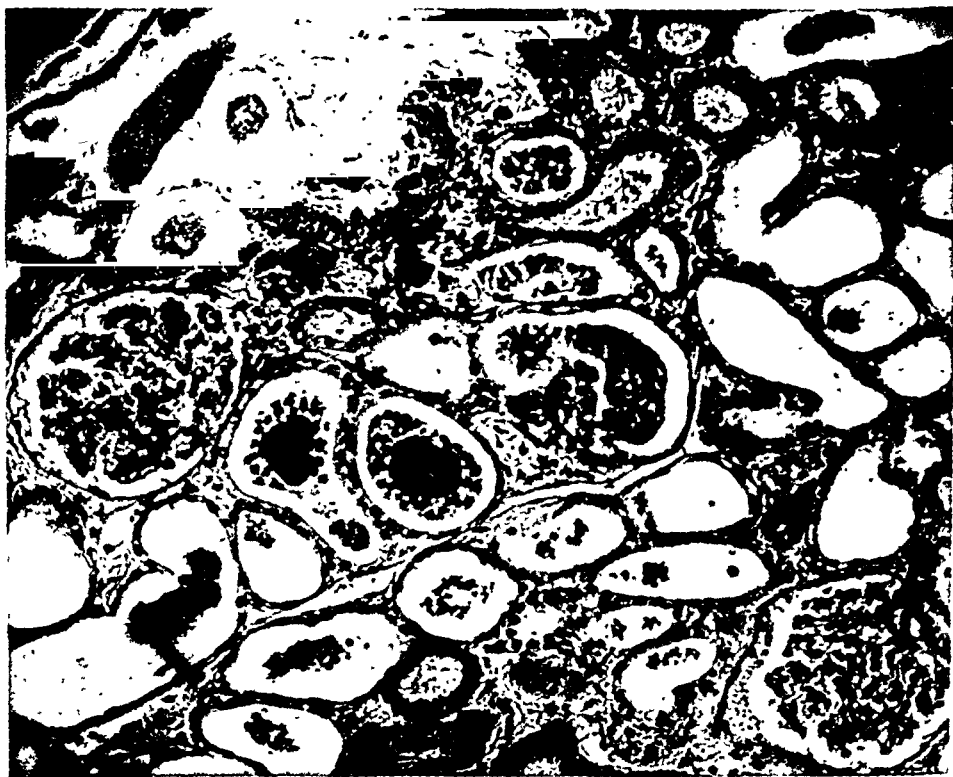


Fig. 3.—Section of kidney shown in Fig. 2. The glomeruli are of normal size and cellularity, but there is protein precipitate within the capsular spaces. The proximal segments are shown in the upper portion and display hyaline droplets in the cytoplasm. The distal segments are lined by thin regenerated epithelium and contain granular heme casts. In the center damaged epithelium has sloughed into the lumen and surrounds a heme cast (brownish granular) in the manner of a collar.

the patient became edematous in the subcutaneous zone, but there was no demonstrable pulmonary edema. Such circumstances can be explained best by considering that water and salts were absorbed through peritoneal surfaces.

The hypertension which began on the third day persisted throughout the irrigation period. During the first three days sodium bicarbonate was given (1 Gm. every four hours) but this was discontinued when the edema was noticed. Within two days a severe acidosis associated with Kussmaul breathing appeared. In required 32 Gm. of sodium bicarbonate in twenty-four hours to alleviate the acidotic breathing. The acidosis was modified but remained severe.



TABLE II (CASE 2)

DAY	BLOOD (MG. %)		CO <sub>2</sub> C.P. (VOL. %)	PLASMA Cl (MEQ./L.)	SERUM Na (MEQ./L.)	SERUM K (MEQ./L.)	ICTERUS INDEX	HB. CONC. (GM. %)	FLUID INTAKE (ML.)	URINE VOL. (ML.)	URINE UREA (GM.)	URINE Cl (NaCl) (GM.)	PERIT. FL. VOL. (ML.)	PERIT. UREA (GM.)	B.P. (MM. HG)
	UREA	N													
1-2	46	99	63	84.5			82.8		4,025	290	1.9	1.1			120/80
3	65	139							400	110	0.7	0.45			120/80
4	100	214	62	86.2	153	3.0	173	9.5	200	115	0.54	0.34	5,100	6.8	
5	107	229	56	71	132	6.2	160	9.5	1,300	225	0.48	0.4	22,200	26.6	
6	101	216	34	82.6			157		2,100	140	0.42	0.5	29,200	25.8	132/84
7	89	191	42	91.3			150		1,450	255	0.6	0.26	27,150	21.8	134/84
8	81	173	22	98	142	4.2	100		2,440	235	0.49	1.1	26,200	14.0	145/75
9	77	165	36	101			92.8	5.6	2,000	300	1.25		20,000	9.0	160/90
10							69	7.2	1,480	250	1.0		20,300	9.0	180/96
11	57	122	26	101			95.7	8.2	1,960	245	0.26	1.2	3,150	2.0	170/90
12	68	145	43	88			97.5	8.6	100	230	0.64	0.86			
13	79	169	38	84.5			111.6		520	620	2.0	2.1			
14	86	184	50	84.5	134	4.9	122.5		1,200	460	1.37	1.7			
15	94	201	43	88					80	475					140/90

The same scheme is used as in Table I. The chloride determination was performed on samples of plasma on each occasion. The normal plasma chloride concentration is usually given as 103 meq. per liter. Notice that a prominent hypochloremia existed at the outset of the irrigation.

Acidosis and increase in the chloride concentration are evident. The fluid intake was accurately measured in this case. After cessation of the irrigation the CO<sub>2</sub> combining power was elevated and the chloride concentration dropped once more.

Up to the fourth day of irrigation there was little evidence of improvement other than reflected by a lowering of the blood urea concentration, a minimal increase in urinary volume, and a recession of the jaundice. At this time pronounced hemodilution existed (hemoglobin 5.6 Gm. per cent) and three small (300 ml.) transfusions were given. That this course was unsuccessful was attested by the recrudescence of the jaundice, the subsequent rather fixed oliguria, progressive weakness, and mental confusion. The course continued downhill with marked gaseous distension, and expiration occurred following a convulsion on the fifteenth day.

On the fifth day of irrigation the outflow fluid became cloudy and showed many strands of fibrin and clumps of leucocytes. A culture at this time yielded a growth of a gram-negative bacillus identified as *Proteus*. Streptomycin therapy was initiated and continued until the exitus (0.25 Gm. intramuscularly every four hours).

Pertinent features of the autopsy findings are given. There was widespread fibrocaseous tuberculosis of the lungs with nearly complete destruction of the right lung and extensive involvement of the left upper lobe; the left lower lobe constituted the only morphologically intact lobe. Of much interest was the complete absence of evident pulmonary edema. A minimal amount of greenish-yellow fibrinopurulent exudate was found about the catheter and drain wounds but in all other areas the peritoneal surfaces were of normal smooth and glistening appearance. The liver displayed moderate fatty metamorphosis and bile staining. The kidneys were outstanding. They were twice normal in weight (right, 300 grams; left, 300 grams); the cut surfaces bulged prominently; the capsule stripped easily, leaving a smooth surface; the cortex was gray-yellow in color; the medulla was dusky and well striated (Fig. 1). Microscopically the following were noted: protein precipitate in the glomerular capsular spaces, normal-appearing glomeruli, prominent hyaline droplet accumulation in the cytoplasm of the cells lining the proximal segments, focal necrosis of the distal segments with focal inflammatory foci (lymphocytes, eosinophils, macrophages, fibroblasts), many heme casts in the distal segments (brownish granular and eosinophilic granular types), prominent sloughing of damaged cells of the distal segment, regeneration of the distal segment, and interstitial edema. The regenerated distal segments were lined by very flat cells as described by Lucke<sup>22</sup> (Fig. 2). Prominent medullary capillary hyperemia was present.

*Comment.*—This case presented several points of much interest. In the first place the exact antigen-antibody mechanism causing the hemolysis was not demonstrated, but undoubtedly multiple hemolytic transfusion responses occurred and resulted in a severe type of renal insufficiency. The failure to demonstrate any type of antibody (agglutinating, blocking, developing) in the patient's serum, despite daily checks, was a new experience for this center. It could be that the transfusions cleared the serum of antibodies and that the widespread tuberculosis hindered further antibody production.

A series of interesting points were related to the irrigating procedure. It was reasoned that by initiating irrigation early it would be possible to keep ahead of the deleterious aspects of renal insufficiency and to precipitate an early recovery of the kidneys. It is now clearly apparent that this was a distinct mistake. The usual progress of the renal phase was not altered, as can be appreciated by comparing this case with fourteen others discussed elsewhere.<sup>11</sup> Considerable urea was cleared from the body, but a severe acidosis developed and generalized edema was a continuous threat. It becomes apparent that the difficulties in this case resembled those of Case 1.

By the foregoing remarks it is not meant to imply that the irrigation procedure necessarily caused the fatality. Two other complications were more likely to have been implicated in this sense: (1) overwhelming pulmonary tuber-

culosis demonstrated at autopsy and (2) repeated transfusions with hemolytic response. But it does appear that the procedure was begun much too early, that the usual course of these cases was not altered, and that certain deleterious complications were once more demonstrated.

Finally, this case offered three other worth-while informative features. (1) The washing out of salts and base via the peritoneum by a nonsalt-containing fluid was again revealed. This is merely a clinical application of the Darrow-Yannet experiment<sup>21</sup> and is fraught with possible deleterious fluid shifts under certain conditions (see Discussion). (2) The absence of a diffuse peritonitis following seven and one-half days of irrigation when penicillin and streptomycin were used is in itself worthy information. (3) The morphologic renal picture corresponded with the lesions designated by Lucke<sup>22</sup> as "lower nephron nephrosis" and included sloughing of damaged tubular epithelium and well advanced regeneration of the same by the fifteenth day.

CASE 3.—This case is that of a colored woman, aged 49 years, who developed a prominent hypotension associated with blood loss during a hysterectomy for multiple leiomyomas.\* Due to the urgency of the hypotension, unmatched blood was given (500 ml.).

The blood pressure remained low (70/40 to 86/70) for nearly twenty-four hours. The patient had received 3,500 ml. of blood. A laparotomy revealed a hematoma in the pelvis and the oozing of blood; this was controlled and the blood pressure returned to normal. (Hemorrhagic tendencies are not an infrequent complication of hemolytic reactions.)

On the second day the icteric index was 26. The first urine specimen was dark red. Oliguria became marked (for ten days about 100 ml. daily). There was mounting azotemia, acidosis, and hypochloremia (Table III). Vomiting was troublesome. The patient became comatose and developed muscular twitching. The total amount of fluid intake is not known. During the first five days roughly 3,000 ml. were given in addition to the blood given for the hypotension. Between the fifth and tenth day 13,500 ml. of intravenous fluids were given, of which 5,000 ml. were of isotonic sodium salt composition.

TABLE III (CASE 3)

DAY	BLOOD (MG. %)		CO <sub>2</sub> C.P. (VOL. %)	BLOOD Cl (MEQ./ L.)	SERUM Na (MEQ./ L.)	PLASMA PROT. CONC. (GM. %)	HB. CONC. (GM. %)	URINE VOL. (ML.)	URINE UREA (GM.)	IC- TERIC INDEX	PERIT. FLUID VOL. (ML.)	PERIT. UREA (GM.)
	UREA N	UREA										
1												
2			55	99			9.8	100?		26		
3								100?				
4								100?				
5	106	227						150?		8		
6	105	225	42					100				
7	168	360	28	86.4		7.1				6		
8	222	475	45.7	72.9	140		11.5	200				
9					140		7.8	100	0.08			
10	198	424	69.8	66.6								
11	220	471	50.4	52.5			6.8	150			17,000	30
12	182	389	50.7	99.9	150	7.6		380	0.9		22,210	22
13	126	270	33.7	116				610	1.36		19,000	19.5
14	106	227		104.7								

\*The chloride concentration is once more as blood chloride (average normal, 84.5 meq. per liter). The total fluid intake was not accurately measured or estimated.

Again the drop in CO<sub>2</sub> combining power of plasma and the increase in chloride and sodium concentration during the irrigation are evident.

\*This case was observed in another hospital by one of us (A. B. S.). It is not included in the fifteen cases of hemolytic reaction reported separately.<sup>11</sup>

During the eighth, ninth, and tenth days the stomach was periodically washed for clearance purposes. It is of interest that on two occasions the urea concentration of the aspirated fluid was determined to be 3.4 and 3.2 mg. per 100 milliliters.\* During this period there was a slight decrease in the blood urea concentration, but the  $\text{CO}_2$  combining power of the plasma was elevated and the hypochloremia became of extreme proportions. The serum sodium concentration was within normal limits.

On the tenth day peritoneal irrigation was started because the patient did not appear to be improving. During the following three days 71.5 Gm. of urea were cleared from the body by this means. The urine volume output was increasing slightly. The blood urea concentration dropped markedly but there was pronounced acidosis (Table III). The blood chloride and serum sodium concentration were prominently elevated. On the fourth day of the irrigation the patient succumbed to an accident not related to the peritoneal irrigation or to the renal insufficiency.† The ultimate outcome of this case, therefore, is not subject to appraisal in so far as the renal lesion and irrigation are concerned.

*Comment.*—Again peritoneal irrigation was responsible for the clearance of a substantial quantity of urea from the body, but concomitantly a severe acidosis developed and there was once more evidence of the uncontrolled absorption from the peritoneal surfaces (elevated Cl and Na concentrations).

The autopsy findings demonstrated typical renal lesions. There were heme casts and sloughing of the damaged cells of the distal tubule and evidence of regeneration by the fourteenth day. Again no diffuse peritonitis was encountered.

TABLE IV. UREA CONCENTRATION (MG. PER 100 ML.)  
BLOOD AND PERITONEAL FLUID

DAY	CASE 1		CASE 2		CASE 3	
	BLOOD	PERIT.	BLOOD	PERIT.	BLOOD	PERIT.
1	310	133.5	214	133	472	176
2	214	103	229	120	389	99.5
3	178	76	217	88.5	269	102.6
4	169	71.2	191	80.2		
5	120	58.4	174	53.4		
6	107	71	165	64.6		
7-8			123	65		

Notice the usual decline in the urea concentration of the peritoneal escape fluid as the blood urea concentration decreased. These concentrations are minor as compared with the normal urine urea concentration.

#### DISCUSSION

Continuous peritoneal irrigation has been demonstrated again to remove urea from the body. The urea has been used as an index of the clearance of other waste products. The degree of efficiency of this procedure can be appreciated by examining Table IV. The highest urea concentration in the outflow fluid occurred on the first day when the blood urea level was highest.‡ This highest concentration still fell short of the normal urine urea concentration by ten to fifteen times. Extremely large volumes of fluid therefore were necessary in order to clear daily between 15 and 30 Gm. of urea from the body. As the blood urea concentration was lowered there was some correlation with the decrease in the peritoneal fluid concentration.

\*At this concentration in order to clear the 30 Gm. of urea removed on the first day of peritoneal irrigation it would have required 1,000,000 ml. of gastric washing fluid.

†While a sternal transfusion was being attempted (hemoglobin, 6.8 Gm. per cent) 3,000 ml. of blood were introduced into the mediastinum and pleural spaces.

‡This feature satisfies the law of partial pressure as applied to osmotic interchanges.

Two major complications were related to the irrigation procedure in each case: (1) a severe acidosis and (2) the absorption of water and salts from the peritoneal surfaces. There were indications that the acidosis was due to the differential washing out of base while neutral salts were absorbed. Generalized edema developed or became accentuated, and the threat of pulmonary edema remained constant.

Peritonitis was demonstrated in each case but was of a focal nature, being localized about the intake catheter and outflow drain. As a result of exudate accumulation about the "sump" drain the recovery of fluid became less and less efficient after the fifth day. Diffuse peritonitis apparently was prevented by penicillin and streptomycin. Gaseous distention was troublesome in one case.

We are familiar with six other cases of peritoneal irrigation for acute renal damage reported in the literature of recent times<sup>7-9</sup>; four of these cases had renal insufficiency following incompatible blood transfusions. The present group brings the total number to seven. In order to appraise this procedure it becomes necessary to review briefly the sequence of events in such cases.

The clinical course of patients experiencing incompatible blood transfusions may be divided into three phases<sup>11</sup>: (1) *hemolysis, hypotension, etc.*, is characterized by a sudden onset with apprehension, tightness in the chest, backache, dyspnea, cyanosis, mental confusion, hypotension, chill-fever, and hemoglobinemia-hemoglobinuria. (2) *Renal insufficiency* is attended by oliguria, heme casts in the urine, hypertension, azotemia, altered chemical pattern of blood (mainly depressed serum sodium, calcium, blood chloride concentration, and CO<sub>2</sub> combining power of plasma), and mounting antibody titer. (3) *Copious diuresis* is accompanied by marked water-salt loss from the body. Experience with these and related cases have led us to the conclusion that each phase requires its own distinctive type of management.<sup>11</sup> During the first phase, blood transfusion of undoubted compatibility should be given to cope with anemia and lowered blood volume, thus decreasing the period of hypotension. During the second phase, forcing fluids seems deleterious because the kidneys are not in a position to handle water and salts. The best results during this period have been obtained by restricting the fluid intake to a conservative estimated insensible loss plus the scanty urinary output (a total usually of 800 to 1,500 ml. daily for six to eight days). A high caloric-low salt formula has been given with this fluid plus water-soluble vitamins. Sodium bicarbonate (4 to 6 Gm.) has been given daily to cope with an excessive lowering of the CO<sub>2</sub> combining power of plasma. This regime is based on the premise that damaged kidneys require time for healing. When the diuresis appears (eighth to fourteenth day) the water-salt loss through this means is replaced. Such losses may require 20 to 40 Gm. of salts daily plus 5,000 to 10,000 ml. of water. As soon as possible the patient is allowed to adjust his own intake and output.

It has been our experience that patients overloaded with water and salt have expired or the onset of diuresis has been delayed (fourteenth to eighteenth day).

Experience with this three-phase management and with the three cases of peritoneal irrigation has led us to a more critical view of the irrigation procedure. The complications of acidosis and water-salt overload occurring during irriga-

tion seem to be the particular deleterious influences that the outlined three-phase management appears to prevent. With the three-phase management the results have been very encouraging<sup>11</sup>; with the irrigation procedure the results have been rather discouraging.

The discouraging features of peritoneal irrigation are not confined to our three cases. A study of the protocols of the two cases presented by Fine, Frank, and Seligman<sup>7</sup> yield evidence of water-salt overload and of no particular shortening of the usual period necessary for recovery. In the case of Reid, Penfold, and Jones<sup>8</sup> it is difficult to accept the interpretation that the removal of 6.4+ Gm. of urea in two days was an effective means of supporting renal function. Moreover, the onset of the diuresis coincided with the usual time for this phase.

The case of Goodyear and Beard<sup>9</sup> had a stated daily intravenous fluid intake of 3,000 milliliters. Over a sixteen-day period such a regime alone is conducive to a fluid overload when renal insufficiency exists. The amount of urea recovered in the outflow fluid within a six-day period amounted to about 15 grams. One wonders how influential was the factor of dilution in the displayed drop in nonprotein nitrogen concentration. The diuresis phase, which was delayed in onset, occurred after the irrigation.

While treating a case with azotemia, the ability of hydremia to lower the concentration of nitrogenous waste products must be kept constantly in mind. Otherwise a spurious sense of improvement may be enjoyed when actually a deleterious water and/or salt overload is in progress.

The complications encountered in the three presented cases during irrigation have ensued while crystalloid solutions approaching the composition of plasma (minus proteins) were being used. A fluid of different composition may prevent these complications but of this we are not aware at present. Hypertonicity of the inflow fluid has been conducive to pain in a few brief trials.

The results herein presented indicate the need for an earlier and sustained replacement of base. Perhaps the measurement of total base in the outflow fluid would indicate the necessary replacement. Even so, the prevention of the absorption of neutral salts and water remains an additional major problem.

The use of 5 per cent dextrose in distilled water appeared beneficial in Case 1 by removing salt from the body. After nineteen days the kidneys were regenerated and ready for the diuresis phase of functioning. It must be emphasized, however, that the removal of salt in this manner when the kidneys are not ready to discard water may be attended by deleterious intracellular water shift as pointed out by Darrow and Yannet.<sup>21</sup>

#### CONCLUSIONS

1. Experience with these patients following incompatible blood transfusion in whom peritoneal irrigation was conducted for the treatment of renal insufficiency according to described techniques has been disappointing. Substantial quantities of urea (and presumably other waste products) were cleared from the body. However, two major complications, accentuation of acidosis and the absorption of water and salts from the peritoneum, seemed related to the use of crystalloid solutions for irrigation. Such complications are considered

deleterious to the progress of these patients during the renal insufficiency phase and may retard the onset of diuresis. In contrast, an outlined "three-phase" regime seems to oppose these particular complications.

2. Diffuse peritonitis was absent in two cases examined post mortem. Presumably penicillin and streptomycin therapy prevented this complication. Vestibular damage was subsequently observed in one case following streptomycin.

3. The data obtained in the cases herein reported appears to agree with those of other cases of incompatible blood transfusion and renal insufficiency which have been subjected to this procedure and reported in the literature. There seems to be disagreement in the interpretation given these data.

#### REFERENCES

1. Abel, J. J., Rowntree, W. G., and Turner, B. B.: On the Removal of Diffusible Substances From the Circulating Blood of Living Animals by Dialysis, *J. Pharmacol. & Exper. Therap.* 5: 275, 1914.
2. Putman, T. J.: The Living Peritoneum as a Dialysing Membrane, *Am. J. Physiol.* 63: 548, 1923.
3. Rhoads, J. E.: Peritoneal Lavage in the Treatment of Renal Insufficiency, *Am. J. M. Sc.* 196: 642, 1938.
4. Additional references in German and American literature quoted by Fine and others.<sup>6, 7</sup>
5. Frank, H. A., Seligman, A. M., and Fine, J.: Treatment of Uremia After Acute Renal Failure by Peritoneal Irrigation, *J. A. M. A.* 130: 703, 1946.
6. Seligman, A. M., Frank, H. A., and Fine, J.: Treatment of Experimental Uremia by Means of Peritoneal Irrigation, *J. Clin. Investigation* 25: 211, 1946.
7. Fine, J., Frank, H. A., and Seligman, A. M.: The Treatment of Acute Renal Failure by Peritoneal Irrigation, *Ann. Surg.* 124: 857, 1946.
8. Reid, R., Penfold, J. B., and Jones, R. W.: Anuria Treated by Renal Recapsulation and Peritoneal Dialysis, *Lancet* 2: 749, 1946.
9. Goodyear, W. E., and Beard, D. E.: The Successful Treatment of Acute Renal Failure by Peritoneal Irrigation, *J. A. M. A.* 133: 1208, 1947.
10. Muirhead, E. E., Small, H. B., and McBride, R. B.: Peritoneal Irrigation for Uremia Following Incompatible Blood Transfusion, Case Report, *Arch. Surg.* 54: 4, 1947.
11. Muirhead, E. E., Hill, J. M., Haberman, S., and Haley, A. E.: Hemolytic Blood Transfusions: Concerning Their Clinical Management and Relation to Other Types of Acute Renal Damage. Unpublished.
12. Burnett, W. E., Rosemond, G. P., and Caswell, H. T.: The Use of the "Sump" Drain in Peritoneal Infection, *S. Clin. North America* 24: 1316, 1944.
13. Taylor, W. F., and Blair, W. M.: Improved Method for Determination of Blood Urea Nitrogen by Direct Nesslerization, *J. LAB. & CLIN. MED.* 17: 1256, 1932.
14. Whitehorn, J. C.: Simplified Method for the Determination of Chlorides in Blood or Plasma, *J. Biol. Chem.* 45: 449, 1921.
15. Sanford, A. H., and Sheard, C.: The Determination of Hemoglobin With the Photo-electrometer, *J. LAB. & CLIN. MED.* 15: 483, 1930.
16. Van Slyke, Donald D., and Cullen, Glenn E.: The Bicarbonate Concentration of the Blood Plasma; Its Significance, and Its Determination as a Measure of Acidosis, *J. Biol. Chem.* 30: 289, 1917.
17. Muirhead, E. E., Hill, J. M., Jones, F., and Marinis, T.: The Determination of Sodium and Potassium Concentration by the Flame Photometer. Unpublished.
18. Race, R. K.: The Rh Genotypes and Fisher's Theory; read before the International Hematology Conference, Dallas, Texas, Nov. 15, 1946.
19. Hill, J. M.: The Complexities of the Rh Problem: Some Suggestions for Clarification, *Amer. J. Clin. Path.* In press.
20. Hill, J. M., and Haberman, S.: Hemolytic Rh Immune Globulins: Evidence for a Possible Third Order of Antibodies Incapable of Agglutination or Blocking. Read before the International Rh Conference, Dallas, Texas, Nov. 15, 1946.
21. Darrow, D. C., and Yannet, H.: The Changes in the Distribution of Body Water Accompanying Increase and Decrease in Extracellular Electrolyte, *J. Clin. Investigation* 14: 266, 1935.
22. Lucke, B.: Lower Nephron Nephrosis, *Mil. Surgeon* 99: 371, 1946.

# SERUM PROTEINS IN ACUTE INFECTIONS

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AS TURBIDOMETRIC methods for the estimation of proteins in biologic fluids have been shown to be unreliable,<sup>1</sup> they have been discarded except for rough approximations. During an investigation of the causes of error, it was observed that in certain acute infections a turbidometric method gave consistently low results for serum albumin when compared with those given by other methods. In the following, these experiments are described and certain conclusions reached concerning the nature of serum proteins in infection.

## METHODS

### *Fractionation of Serum Proteins.*—

1. Howe's<sup>2</sup> method. The globulin in 0.5 ml. serum was precipitated with 9.5 ml. sodium sulfate (22.2 Gm. per 100 ml.) at 37° C. and separated by filtration.

2. The Aqueous-methanol method of Pillemer and Hutchinson.<sup>3</sup> The globulin in 1 ml. serum was precipitated by 4 ml. methanol reagent at 0 to 1° C. and separated by centrifuging or by filtration. The clear solution was diluted to four times its volume with saline.

### *Protein Estimations.*—

1. Macro-Kjeldahl nitrogen estimation.

2. Biuret method. The color, developed by Fine's<sup>4</sup> method, was estimated in a photoelectric absorptiometer using a green filter (wave length 525 mμ). The instrument was calibrated from the results of parallel Kjeldahl estimations.

### *Turbidity Methods.*—

1. Total serum protein. One milliliter serum was diluted to 100 ml. with saline (0.9 Gm. per 100 ml.). To 1 ml. of this solution 1 ml. water and 3 ml. sulfosalicylic acid (5 Gm. per 100 ml.) were added.

2. Serum albumin. To 0.5 ml. albumin solution (Howe's filtrate or diluted methanol filtrate), 1.5 ml. water and 3 ml. sulfosalicylic acid solution were added. When the serum albumin was high, the filtrate containing the albumin was first diluted with an equal volume of the solution used for precipitation.

3. For the determination of the specific turbidity (discussed later) of serum globulin the precipitated globulin was washed twice with the precipitant used, dissolved in saline or weak soda, made up to twenty times the original volume of serum, and the protein content and sulfosalicylic acid turbidity estimated. For the latter, 0.5 ml. solution was taken and the precipitation completed as in the case of albumin.

The sulfosalicylic acid was always added slowly, with constant shaking, and the turbid solutions allowed to stand in the incubator for thirty minutes at 37° C. After thorough mixing the optical density of the suspensions was estimated with a photoelectric absorptiometer which had been calibrated with a standard suspension of formazine.<sup>5</sup> This was prepared by mixing 14.5 ml. hexamine solution (10 Gm. per 100 ml.) with 14.5 ml. hydrazine sulfate solution (1 Gm. per 100 ml.) and, after forty-eight hours, diluting to 200 ml. with water. The optical densities of dilutions of this suspension were determined in the absorptiometer, and a curve was constructed from which protein turbidity could be read in terms

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of the standard formazine solution taken as 100. The result was multiplied by a factor depending on the amount of serum present in the aliquot used, so that the final figure represented the turbidity produced in a volume of 5 ml. by 1 ml. of 1 per cent solution of the original serum (total protein, 1; albumin, 0.4, if 0.5 ml. albumin filtrate had been used; globulin, 0.4). For all results recorded here a green filter was used (525 m $\mu$  wave length).

As there are not large differences between the quantities of protein precipitated on each occasion, a comparable figure may be obtained for each protein by dividing the turbidity figure by the percentage of that protein in the serum. This figure will be referred to as the "specific turbidity" of the protein.

# EXPERIMENTS WITH HOWE'S METHOD OF FRACTIONATION OF SERUM PROTEINS

Sera from normal subjects and from patients with acute infections were fractionated and the total protein, albumin, and globulin determined by the biuret method. The turbidity produced in these fractions by sulfosalicylic acid was measured and the specific turbidity of each protein calculated. In a number of cases serum protein values determined by Kjeldahl were used; these always gave similar results.

TABLE I. SPECIFIC TURBIDITY OF SERUM PROTEINS FRACTIONATED BY HOWE'S METHOD (NORMAL AND ABNORMAL CASES)

CASE	SERUM PROTEINS				SPECIFIC TURBIDITY		
	TOTAL (GM./100 ML.)	ALBUMIN (GM./100 ML.)	GLOBULIN (GM./100 ML.)	ALBUMIN GLOBULIN	TOTAL PROTEIN	ALBUMIN	
1	6.8	3.8	3.0	1.3	6.7	9.6	Normal
2	7.0	4.0	3.0	1.3	6.6	9.5	Normal
3	7.4	4.4	3.0	1.5	6.2	8.5	Normal
4	7.4	4.7	2.7	1.7	6.7	7.9	Normal
5	6.9	4.6	2.3	2.0	6.9	8.3	Normal
6	7.7	5.4	2.3	2.3	6.7	8.2	Normal
7	7.8	5.0	2.8	1.8	6.3	8.9	Normal
8	7.0	4.6	2.4	1.9	6.7	8.5	Normal
9	7.4	5.1	2.3	2.2	6.6	8.7	Normal
10	7.0	4.4	2.6	1.7	6.4	8.4	Normal
Average of normals					6.6	8.7	
11	6.8	3.3	3.5	0.9	5.7	5.7	Lobar pneumonia: Fever 5 days
12	6.8	4.0	2.8	1.4	6.1	5.8	Lobar pneumonia: Fever 4 days
13	7.8	4.6	3.2	1.4	5.3	5.2	Lobar pneumonia: Fever 6 days
14	5.3	2.9	2.4	1.2	5.7	5.8	Lobar pneumonia: Fever 2 days
	6.3	3.8	2.5	1.5	6.1	7.4	Afebrile 16 days
15	4.2	2.3	1.9	1.2	6.0	3.2	Lobar pneumonia: Fever 7 days
16	7.2	3.7	3.5	1.1	6.0	6.2	Appendix abscess
	7.9	4.1	3.8	1.1	5.3	6.9	8 days after drainage
	8.5	4.2	4.3	1.0	6.6	7.8	20 days after drainage
17	6.8	3.3	3.5	0.9	5.4	4.9	Infected hydatid cyst; lung
18	6.5	3.5	3.0	1.2	6.0	5.0	Infected hydatid cyst; lung
	6.5	3.4	3.1	1.1	5.1	4.6	4 days after drainage
	7.6	4.1	3.5	1.2	5.3	6.2	10 days after drainage
19	5.3	2.5	2.8	0.9	6.3	5.3	Amoebic abscess; liver
	5.8	2.9	2.9	1.0	6.0	6.4	Treatment 10 days
	6.8	3.3	3.5	0.9	6.0	7.2	Treatment 17 days
	6.7	3.9	2.8	1.4	5.9	7.1	Treatment 24 days
	7.0	4.2	2.8	1.5	5.8	7.6	Treatment 50 days
20	7.0	3.5	3.5	1.0	4.9	3.6	Amoebic abscess; liver
21	7.0	3.2	3.8	0.8	5.4	5.7	Osteomyelitis; femur
22	6.8	2.5	4.3	0.6	4.9	6.8	Osteomyelitis; face
Average of abnormals (Initial values only)					5.6	5.3	

*Results.*—From Table I it can be seen that in acute infections the specific turbidity of total protein and of albumin is reduced, the reduction being small in the case of total protein, large in the case of albumin. During convalescence the value of the latter returns toward normal.

The specific turbidity of globulin in normal persons and in patients with acute infection is given in Table II. It is low and nearly constant and shows no change parallel to that of the albumin. This suggests that the reduction in the specific turbidity of the total protein and the much greater reduction in that of the albumin is to be sought in a change in the albumin-sulfosalicylic acid precipitate. Such a change might be chemical and/or physical; if physical, it might be brought about by the presence of some substance in the albumin filtrate.

In a number of cases the albumin in the filtrate was precipitated by 9 volumes of hot acetone-ethanol (50:50), centrifuged, the supernatant fluid poured off, and the precipitate, after being drained, dissolved in weak caustic soda solution. The turbidity produced by sulfosalicylic acid was determined and the

TABLE II. COMPARISON OF SPECIFIC TURBIDITIES OF HOWE'S ALBUMIN AND GLOBULIN FRACTIONS (NORMAL AND ABNORMAL CASES)

CASE	23	24	25	26	27	28	29	30	31	32	33	34	AV.
Specific turbidity of albumin	8.7	8.3	8.3	8.2	7.8	7.6	7.5	6.8	6.3	6.2	5.7	5.2	--
Specific turbidity of globulin	4.6	3.9	4.0	4.7	4.8	4.5	3.9	4.1	4.4	4.4	4.4	4.2	4.3

TABLE III. COMPARISON OF SPECIFIC TURBIDITIES OF HOWE'S ALBUMIN FRACTION BEFORE AND AFTER PRECIPITATION WITH ACETONE-ETHANOL (NORMAL AND ABNORMAL CASES)

CASE	35	36	37	38	39	40	41	42	43	44	45	46
Specific turbidity of albumin	8.5	8.0	7.9	7.9	6.9	6.4	6.1	5.7	5.3	5.0	4.9	4.8
Turbidity (No precipitation)												
Turbidity (Precipitation)	1.3	1.4	1.3	1.3	1.4	1.3	1.2	1.4	1.3	1.3	1.5	1.2

TABLE IV. COMPARISON OF ALBUMIN FRACTION SEPARATED BY HOWE'S METHOD AND BY AQUEOUS-METHANOL METHOD (NORMAL AND ABNORMAL CASES)

CASE	SERUM ALBUMIN (GM./100 ML.)		(1) - (2)	(1) - (2)	ALBUMIN SPECIFIC TURBIDITY	
	SODIUM SULFATE (1)	AQUEOUS METHANOL (2)		(1) × 100 (3)	SODIUM SULFATE	AQUEOUS METHANOL
47	4.9	4.2	0.7	14	8.9	11.1
48	5.1	4.4	0.7	14	8.7	11.9
49	5.2	4.7	0.5	9	8.6	10.6
50	4.6	3.8	0.8	17	8.4	12.0
51	3.4	2.4	1.0	29	7.8	11.0
52	3.5	2.4	1.1	31	7.8	12.2
53	3.9	2.8	1.1	27	7.5	12.5
54	3.8	2.8	1.0	26	7.4	12.8
55	2.9	1.9	1.0	34	5.8	11.7
56	2.6	1.6	1.0	38	5.0	11.9
57	3.3	2.0	1.3	40	5.0	11.5
58	4.0	2.1	1.9	47	4.4	12.8
Average						11.8

specific turbidity calculated and compared with that of albumin from the same sera without acetone-ethanol precipitation. The figures in Table III show that, while there is a difference in the specific turbidities of albumin which has and which has not been precipitated by acetone-ethanol, these values bear a constant relationship to one another. This indicates that the cause of the low specific turbidity of albumin in acute infections cannot be separated from the albumin by acetone-ethanol and that it is due to a change in the precipitate itself. Such a change might either be the precipitation of varying amounts of some other substance (for example, carbohydrate or lipid on the albumin-sulfosalicylic acid precipitate) or the presence in the albumin filtrate in varying proportions of two or more protein substances having different specific turbidities. Lipid contamination is unlikely to be the explanation since acetone-ethanol precipitation would probably remove much of this and the relationship apparent in Table III would not exist.

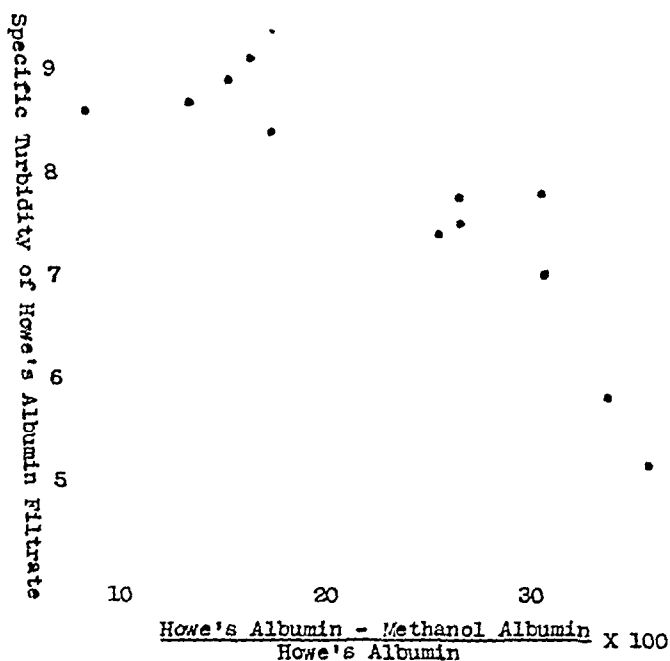


Fig. 1.

#### EXPERIMENTS WITH AQUEOUS-METHANOL FRACTIONATION OF SERUM PROTEINS

Pillemer and Hutchinson<sup>3</sup> have shown that their method of fractionation gives an albumin:globulin ratio which agrees more closely with that obtained by electrophoresis than does Howe's method. Table IV gives the results of simultaneous serum albumin estimations by the two precipitation methods together with the specific turbidities of both albumin fractions.

It will be seen that sodium sulfate invariably precipitates less protein than does the methanol reagent, a result noted by Pillemer and Hutchinson. The difference is small in normal cases (Cases 47 to 50), larger in cases of acute in-

fection, and represents protein precipitated by the methanol reagent but not by sodium sulfate. If this portion is calculated as a percentage (Table IV, column 3) of Howe's albumin, of which it forms a part, it is seen to vary closely with the specific turbidity of the latter (see Fig. 1).

When the last two columns of Table IV are compared, the specific turbidity of albumin separated by the methanol reagent is seen to be high and, unlike that of Howe's albumin, not to fall in infection. The specific turbidity of methanol globulin was determined in a few cases and found to be low (like Howe's globulin), having a value of 4.7.

Three simple fractions are thus distinguishable: (1) a methanol albumin fraction of high specific turbidity; (2) Howe's globulin fraction of low specific turbidity; and (3) a fraction present in Howe's albumin because not precipitated by sodium sulfate, and also present in methanol globulin because precipitated by the methanol reagent. Since the specific turbidity of methanol globulin is low, this third fraction must have a low specific turbidity too. The reduced specific turbidity of Howe's albumin is therefore explained by the presence in it of this material having a low specific turbidity.

#### COMMENT

Normally the amount of the third fraction is small, but in certain acute infections it may amount to as much as 40 to 50 per cent of Howe's albumin. Since the change is accomplished within a day or two of the onset of the infection and coincides with a sudden drop in methanol albumin, it is reasonable to suppose that it is formed by the modification of part of the albumin. This "modified albumin," like globulin, is precipitated by the methanol reagent and has a low specific turbidity; but like albumin, it is not precipitated by a 22.2 per cent solution of sodium sulfate.

Tillett and Francis<sup>6</sup> have demonstrated by bacteriologic methods the presence of a nonspecific protein substance, called by them C protein, in the serum of patients with acute infections. This substance was shown by Abernethy and Avery<sup>7</sup> to be associated with the albumin fraction when globulin is precipitated by ammonium sulfate, while by electrophoresis Perlman, Bullowa, and Goodkind<sup>8</sup> have found it in the alpha-1 globulin fraction. These observations suggest that C protein might be the same as the modified albumin described in the foregoing. Modified albumin, however, is not precipitated by 50 to 70 per cent saturation with sodium sulfate as is C protein, according to MacLeod and Avery.<sup>9</sup> Löfström<sup>10</sup> has produced evidence that his nonspecific capsular swelling substance is identical with C protein, but further work is required to confirm the strong presumption that these three substances are one and the same.

#### SUMMARY

1. In the serum of patients with certain acute infections, a protein is present which differs from normal serum albumin and serum globulin.
2. It appears during the first few days of the infection and is probably formed from serum albumin.

3. This "modified albumin" resembles serum albumin in that it is not precipitated by a 22.2 per cent sodium sulfate solution, but resembles serum globulin in that it is precipitated by a suitable strength of methanol in water.

4. The production of turbidity with sulfosalicylic acid and the estimation of the "specific turbidity" can be used in the investigation of a protein.

## REFERENCES

1. Plimmer, R. H. A., and Lowy, L.: Comparison of Methods of Estimating Blood Proteins, *Biochem. J.* 39: 17, 1945.
2. Howe, P. E.: The Use of Sodium Sulphate as the Globulin Precipitant in the Determination of Proteins in Blood, *J. Biol. Chem.* 49: 93, 1921.
3. Pillemer, L., and Hutchinson, M. C.: The Determination of the Albumin and Globulin Contents of Human Serum by Methanol Precipitation, *J. Biol. Chem.* 158: 299, 1945.
4. Fine, J.: The Biuret Method of Estimating Albumin and Globulin in Serum and Urine, *Biochem. J.* 29: 799, 1935.
5. King, E. J., and Haslewood, G. A. D.: Permanent Standards for Turbidimetric Estimation of Protein, *Lancet* 2: 1153, 1936.
6. Tillett, W. S., and Francis, T. J.: Serological Reactions in Pneumonia With a Non-Protein Somatic Fraction of *Pneumococcus*, *J. Exper. Med.* 52: 561, 1930.
7. Abernethy, T. J., and Avery, O. T.: The Occurrence During Acute Infection of a Protein Not Normally Present in the Blood (I), *J. Exper. Med.* 73: 173, 1941.
8. Perlman, E., Bullova, J. G. M., and Goodkind, R.: An Immunological and Electrophoretic Comparison of the Antibody to C. Polysaccharide and the C-Reactive Protein of Acute Phase Serum, *J. Exper. Med.* 77: 97, 1943.
9. MacLeod, C. M., and Avery, O. T.: The Occurrence During Acute Infection of a Protein Not Normally Present in the Blood (II), *J. Exper. Med.* 73: 183, 1941.
10. Löfström, G.: Comparison Between the Reactions of Acute Phase Serum With *Pneumococcus* C-Polysaccharide and With *Pneumococcus* Type 27, *Brit. J. Exper. Path.* 25: 21, 1944.

## LABORATORY METHODS

### A SIMPLIFIED METHOD FOR THE PRESERVATION OF BACTERIA BY DESICCATION IN VACUO

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WITH THE TECHNICAL ASSISTANCE OF ELSIE LANKFORD

THE presence of water in liquid form being a *sine qua non* of active life, the removal of water or, what is almost equivalent from a physiologic standpoint, its immobilization by freezing puts an end to detectable physiologic activity. Life, however, or at least viability, is not necessarily terminated, and this fact is the basis of several methods for the preservation of bacteria in an inactive condition for long periods. Probably the earliest use of this principle was made by Shattuck and Dudgeon in 1912.<sup>1</sup> Processes for preserving bacteria by desiccation after freezing were described by Rogers<sup>2</sup> in 1914 and by Swift<sup>3</sup> in 1921. Brown<sup>4-7</sup> devised a simpler method using only desiccation in vacuo at room temperature, and first showed one of us (M. F., Jr.) how to use it. Brown<sup>7</sup> reported the survival of streptococci, diphtheria bacilli, pneumococci, and other bacteria for periods of from four to twelve years. A modification of his process was described by Leitson<sup>8</sup> in 1936. There is evidence<sup>||</sup> to indicate that the principal function of the vacuum in these processes is that of speeding desiccation, which in turn stops autolysis and other intracellular processes which would prove lethal, although withdrawal of oxygen also doubtless reduces the rate of death of the desiccated cells. Any factors, such as higher and more rapidly produced vacuum, thinner layers of fluid, more effective dehydrating agents, etc., which speed up removal of water, add to the efficacy of the method and probably permit prolonged survival of the more delicate organisms.

In a study of *Serratia marcescens*, Naylor and Smith<sup>9</sup> have shown that high percentages (99 per cent) of cells survive lyophilization if aerated, young cultures are suspended in a solution containing ascorbic acid, thiourea,  $\text{NH}_4\text{Cl}$ , and dextrin at pH 6 to 7. A high vacuum is necessary to high percentage

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||The older preparations of bacteria listed in this paper were found by one of us (M. F., Jr.) in 1930 to be viable in jars in which the original vacuum no longer persisted after several years of storage at room temperature and transoceanic shipment, unrefrigerated, lasting several weeks in tropical waters. These same jars of cultures, with the same vials, are included in this latest (1946) series of viability tests.

of survival. Attention is called also to the work of Stark and Herrington<sup>10</sup> who published data on differences in resistance of various species to drying in vacuo. Their remarks on free and bound water are of interest. Rahn<sup>11</sup> has given an excellent review of data on the susceptibility and fate of bacteria under desiccation and a discussion of the protective effect of various suspending materials. It seems to be generally agreed that organic colloids have a protective action as a rule, while, in general, contact with oxygen lessens the survival time of the bacteria. In the present study no attempt was made to obtain data on numbers of bacteria surviving after various intervals.

Several processes are in current use in which intracellular water is first immobilized by instantaneous freezing. The effect of this rapid freezing is vitrification rather than crystallization.<sup>12</sup> The water is afterward removed by producing a vacuum in the vessels containing the frozen material. This results in sublimation.<sup>13, 14</sup> The basic principle of this process was used by Sawyer and co-workers<sup>15</sup> for the preservation of yellow fever virus in their early studies, and it proved highly effective. Instantaneous freezing followed by storage at extremely low temperatures ( $-76^{\circ}\text{C.}$ ) was shown by Turner and associates<sup>16, 17</sup> to be a most effective means of preserving living *Treponema pallidum* and other spirochetes, as well as viruses, in status quo for long periods. More recent studies have shown the applicability of freezing as a means of preserving protozoa and metazoa.<sup>18</sup>

The methods of Swift, of Brown, and of Leifson are not, so far as we are aware, satisfactory for long-time preservation of spirochetes and viruses, as is the method of Turner and co-workers, but a wide variety of other organisms may be preserved by desiccation in vacuo for many years without apparent loss of virulence or alteration of other important properties such as colonial form; motility; hemolysis, pigment or toxin production; morphology; staining and fermentative characters. Although a few reports of remarkable longevity of bacteria on ordinary culture media are to be found in the literature,<sup>19, 20</sup> the usual experience is that stock cultures die off in a few months if not transferred to fresh media. This is inconvenient, time consuming, and expensive and induces alterations in the bacteria, as well as contamination.

This communication is made to describe a simplified method for preservation by desiccation in vacuo, modeled after that of Brown, and to show what may be expected of this simplified method. No claim to greater perfection of final vacuum or permanence of closure is made. The principal advantages are simplicity, availability, effectiveness, and low cost under a variety of conditions. The only special apparatuses needed are a good vacuum pump, such as the "Cenco Hyvac," and a Pyrex vacuum desiccator provided with a one-hole rubber stopper connected to the pump. A one-pint Mason fruit jar, about 100 Gm. of  $\text{CaCl}_2$ , and 1 ounce of plasticine are the only materials needed for the desiccation. The bacteria are placed, suspended in sterile rabbit blood, in shell vials 8 by 25 mm.,\* previously somewhat less than half filled with

\*Arthur H. Thomas Co., Catalogue No. 3648.

"washed and ignited" sea sand, plugged with cotton, and sterilized (Fig. 1, A). A good supply of these vials prepared ahead of time makes the preservation of bacteria in vacuo very simple indeed. The necessary steps are as follows:

#### PREPARATION OF CULTURE

1. The desired organism is cultivated on an agar slant of appropriate composition. After good growth has developed, about 0.5 ml. of sterile, defibrinated or citrated rabbit's blood is placed on the slant and the growth is scraped off the agar and emulsified in the blood. This yields a much heavier suspension than would be obtained by desiccating a broth culture, and the blood, it is believed, when dried on the bacteria, forms a protective coating around them. Heller<sup>21</sup> has recently shown blood to be the best material for desiccation.

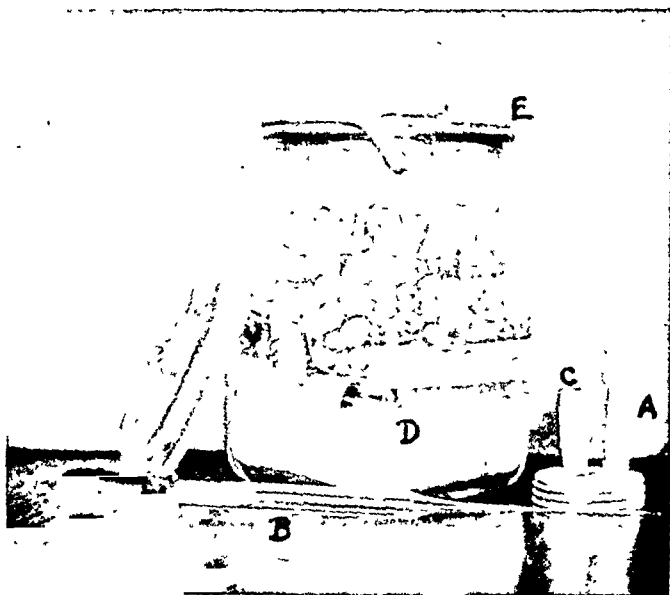


Fig. 1.—A, Sterile vial; B, pipette; C, blooded vial; D, cotton; E, plasticine.

2. A capillary pipette with 1 ml. rubber bulb (Fig. 1, B) is used to take up the blood and transfer it to a sterile shell vial containing sand. The tip of the pipette containing the blood is gently worked downward through the sand until it is at the bottom of the vial and the blood is then made to rise slowly through the sand, thoroughly wetting it by moving the tip of the pipette about gently. Keeping the tip of the pipette at the bottom of the vial, one now withdraws as much of the blood as possible, leaving the sand grains coated with the bacteria-bearing blood. If too much loose blood is left in the vial, it forms a hard, solid mass when dried, which is very difficult to break up when making subcultures from the vial. It is well, therefore, to separate the sand grains somewhat by pushing them partly up the inner side of the vial with the tip of the pipette before finally removing it, so that the grains will not be glued together in a solid mass but can be taken out in small clumps



for subculture. When the sand has thus been "blooded" and the excess blood removed, the cotton plug is replaced.

3. The plug is pushed down in the vial until the upper half of the vial is full of cotton. With scissors, the protruding portion of the plug is clipped off and the tip of the vial is singed so that no cotton fibers protrude (Fig. 1, *C*). Otherwise they may cling to various objects and be pulled out. India ink labelling, covered with nail polish, is convenient. Adhesive tape may also be used as labels. Gummed paper labels are unsatisfactory.

#### *Desiccation.*—

1. In the Mason jar, from which the wire clamp has been removed, place about 100 Gm. of technical or C.P. granular calcium chloride. Over this place a ball of cotton sufficient to hold the calcium chloride in place (Fig. 1, *D*). On the cotton place the labelled vials.



Fig. 2.—Type of desiccator and arrangement of moisture traps and manometer.

2. Roll some of the plasticine on the table into a cylinder with the diameter of a lead pencil (about 8 to 10 mm.) and just long enough to form a complete circle around the uppermost ledge of the jar. It is convenient to determine the proper length for the cylinder of plasticine beforehand by making two marks on the table.

3. Put the plasticine in place on the jar (Fig. 1, *E*) and push it into the depression around the top, molding it about 5 to 8 mm. above the top and flaring slightly outward. Place the lid on the plasticine and press it down so that the bottom of the rim of the lid is about 2 mm. below the level of the topmost rim of the jar. Smooth the plasticine all around and make sure that the closure is complete.

4. Insert a needle through the plasticine seal, pointing it slightly upward so that it will pass between the rim of the jar and the lid. This leaves an opening about 0.5 mm. in diameter for the escape of air. Be sure the hole is patent.

5. Place the Mason jar inside the vacuum desiccator, close the desiccator tightly, and draw the highest vacuum possible (Fig. 2). It should be at least within 0.1 mm. of barometric pressure. A folded towel should line the desiccator to prevent breakage should the jar topple over.

6. Stop the vacuum pump and, with a *sudden* jerk, withdraw the stopper of the desiccator so that there is a quick, copious inrush of air. The lid of the Mason jar is thus pressed down, occluding the needle hole and firmly squeezing the plasticine ring. The vacuum in the jar will be within a few millimeters of that originally present. This may be verified if desired by including a small, siphon, mercury barometer in the Mason jar before closure. They are not essential but are a useful guide at first. The construction and use of these barometers were described by Brown<sup>4</sup> and Leifson.<sup>8</sup>



Fig. 3.—Method of handling knife in opening the evacuated jar.

#### *Recovery of the Culture.*—

1. A tube of broth is prepared by adding to it a drop of sterile defibrinated blood and a drop of sterile 10 per cent aqueous dextrose solution. Other media, appropriate to the bacteria involved, may be used.

2. The effect of the vacuum in the jar is felt when an attempt is made to remove the cover. Force the blade of a large, strong knife (using the portion of the blade close to the handle) between the edge of the lid and the shoulder of the jar. Rotate the knife on its long axis, lowering the back of the knife and keeping the blade firmly pressed between cover and jar (Fig. 3). A screw-

driver blade can also be used but may chip or crack the lid. A sudden hiss signalizes the destruction of the vacuum. Having removed the lid, scrape the plasticine together to smooth the surfaces and to use again.

3. In preparing to recover the culture, partially remove the cotton stopper from the vial with a fine-pointed forceps. After this, handle the cotton stopper as though it were in a diminutive culture tube.

4. A fairly rigid wire is used to loosen the sand grains and to transfer a few of them from the vial to the broth. A two-inch length of No. 14 iron wire or an unfolded paper clip fastened to a needle holder will serve this purpose very well. After flaming the wire, allow a few moments for it to cool; then plunge it first into the tube of broth to be used for the subculture. This cools and wets it.

5. Then introduce the wetted wire into the shell vial. By moving it about, a few grains of sand are loosened. These adhere to the wet wire and are transferred to the blood-dextrose broth. It is best to transfer about a dozen grains.

6. The plug is replaced in the vial and it is resealed in the Mason jar as described. The blood-dextrose broth is incubated appropriately. Occasionally a longer-than-usual period of incubation (for example, forty-eight to seventy-two hours) is necessary.

#### RESULTS

During the period from 1925 to 1928 and at various subsequent times, one of us (M. F., Jr.) preserved, by the method described, several hundred cultures of various species of bacteria. Many of these were taken from the vacuum desiccating jars and tested for viability and cultural properties in 1930 and 1936, but no report was made on the results of those studies. The present report includes the 1936 data and also results of tests of the same identical cultures, and of some others, made in 1946. Thus information is available on the survival of several species of bacteria when desiccated in vacuum for periods ranging from about one to approximately twenty-one years.

The data may be briefly summarized as follows: of 42 strains of beta type hemolytic streptococci, only 6 failed to grow after eighteen years. All but 2 of 20 strains of *Brucella bronchiseptica* grew after nine years and 1 lived seventeen years. Four strains of alpha type streptococci lived five years; 3 tested after eighteen years were not viable. Of 8 strains of pneumococci, 5 strains lived five years, 1 for nine years, 2 were not viable after nine and seventeen years, respectively. Six strains of *Streptococcus faecalis* and *Streptococcus liquefaciens* lived seven to nineteen years.

Of 69 strains of the enteric group, survival of *Eberthella typhosa* and *Salmonella* and *Shigella* species was generally noted after seven years, many strains survived nine years, and 1 strain of *Eberthella typhosa* was alive after eighteen years. *Proteus*, *Aerobacter*, and *Escherichia* and paracolon strains in general lived three to nine years. All of 24 *Staphylococcus* strains were viable after nine years and all of 15 tested survived nineteen years. Hundreds of strains of *Corynebacterium diphtheriae* of all types have survived thirteen years.

Genera generally surviving at least five years are *Serratia*, *Microbacterium*, *Pseudomonas*, *Gaffkya*, *Brucella abortus* (1 strain only four years), *Spirillum rubrum*, and *Lactobacillus*. Species not surviving one year were pathogenic *Neisseria* (six weeks to three months), *Brucella melitensis* and *Brucella suis* (three months), *Hemophilus pertussis* and *Hemophilus influenzae* (six weeks or less). One strain of *Parapertussis bacilli* tested after six weeks was found viable.

It may be said of all surviving organisms that the principal distinguishing characters remained unaltered in any respect. Apparently these organisms had been wholly unaffected by their long dormancy.

It should also be emphasized that, with the few exceptions noted, all of the organisms listed here will remain viable without difficulty for at least a year, thus tiding over intervals between class demonstrations very conveniently. They also remain viable in spite of repeated opening and reclosing of the jars. These facts are perhaps of more practical interest than the survival of some species for twenty-one years or more. When stock cultures are constantly in use, it is more convenient, of course, to maintain them on slants in a vegetative state.

#### SUMMARY

A method of preservation of bacteria in vacuo with simple materials is described. It is evident that, for many species, the method is a desirable substitute for maintenance of vegetative stock cultures and has many important advantages, such as simplicity, economy of time, labor, refrigerator space, and materials, as well as stability and portability of the cultures. A few species, such as pathogenic *Neisseria* and *Hemophilus*, did not survive long under the conditions described, but many others survived for periods ranging from one to twenty-one years.

#### REFERENCES

1. Shattuck, S. G., and Dudgeon, L. S.: Certain Results of Drying Non-Sporing Bacteria in a Charcoal, Liquid-Air Vacuum, *Proc. Roy. Soc., London*, sB. 85: 127, 1912.
2. Rogers, L. A.: The Preparation of Dried Cultures, *J. Infect. Dis.* 14: 100-123, 1914.
3. Swift, H. F.: Preservation of Stock Cultures of Bacteria by Freezing and Drying, *J. Exper. Med.* 33: 69, 1921.
4. Brown, J. H.: The Preservation of Bacteria in vacuo, *Abstracts Bact.* 9: 8, 1925.
5. Brown, J. H.: Vacuum Tubes for the Storage and Shipment of Bacteria, *Science* 64: 429, 1926.
6. Brown, J. H.: Some Laboratory Uses for Crown Seal Milk Bottles, *Science* 74: 391, 1931.
7. Brown, J. H.: The Preservation of Bacteria in vacuo, *J. Bact.* 23: 44, 1932.
8. Leifson, E.: The Preservation of Bacteria by Drying in vacuo, *Am. J. Hyg.* 23: 231, 1936.
9. Naylor, H. B., and Smith, P. A.: Factors Affecting the Viability of *Serratia marcescens* During Dehydration and Storage, *J. Bact.* 52: 565, 1946.
10. Stark, C. N., and Herrington, B. L.: The Drying of Bacteria and the Viability of Dry Bacterial Cells, *J. Bact.* 21: 13, 1931.
11. Rahn, O.: Physical Methods of Sterilization of Microorganisms, *Bact. Rev.* 9: 1, 1945.
12. Weiser, R. S., and Hargiss, C. O.: Studies on the Death of Bacteria at Low Temperatures. II, *J. Bact.* 53: 71, 1946.
13. Florsdorf, E. W., and Mudd, S.: An Improved Procedure for Preservation of Sera, Microorganisms and Other Substances. The Chrycochem Process, *J. Immunol.* 34: 469, 1938.
14. Taylor, A. R., and Beard, J. W.: An Inexpensive Apparatus for Drying From the Frozen State, *Science* 92: 611, 1940.
15. Sawyer, W. A., Lloyd, W. M., and Kitchen, S. F.: The Preservation of Yellow Fever Virus, *J. Exper. Med.* 50: 1-13, 1929.

16. Turner, T. B.: The Preservation of Virulent *T. pallidum* and *T. Pertenu* in the Frozen State With a Note on the Preservation of Filterable Viruses, *J. Exper. Med.* 67: 61, 1938.
17. Turner, T. B., and Fleming, W. L.: Prolonged Maintenance of Spirochaetes and Viruses in the Frozen State, *J. Exper. Med.* 70: 629, 1939.
18. Weinman, D., and McAllister, J.: Prolonged Storage of Human Pathogenic Protozoa With Conservation of Virulence, *Am. J. Hyg.* 45: 102, 1947.
19. Sears, H. J.: Survival for Fourteen Years on Agar Slant Cultures of *Escherichia coli-mutabile* Without Loss of Important Characters, *J. Bact.* 51: 4, 1946.
20. Francis, E.: Twenty-Year Survival of Virulent *Bacillus pestis* Cultures Without Transfer, *Pub. Health Rep.* 58: 1379, 1943.
21. Heller, C.: A Quantitative Study of Environmental Factors Involved in Survival and Death of Bacteria in the Desiccated State, *J. Bact.* 41: 2, 1941.

## DESCRIPTION OF APPARATUS AND METHOD FOR OBTAINING NASAL WASHINGS

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AT THE beginning of an investigation of the etiology of minor upper respiratory diseases, it became evident that a new method was required for obtaining nasal washings. After several attempts at collecting washings by the usual method (instilling material into the nostrils, gargling, and expectorating), the unpleasantness experienced by the donor was of sufficient magnitude to warrant investigation of a new method.

It is known that the anatomy of the nose and nasopharynx is such that material instilled into one nostril can be evacuated from the other by suction without interfering with oral respiration. This is the basis for the "nasal douche with suction," "Nichols nasal siphon," and similar therapeutic aids which have enjoyed varying popularity for giving comfort to individuals with obstructed upper respiratory passages. Such procedures usually require the patient to close off the posterior nasal space by elevating the soft palate and uvula against the posterior pharyngeal wall. However, with the subject supine and head extended 20 degrees or more (Fig. 1), fluid cannot readily pass into the larynx and trachea, despite the position of the soft palate and uvula, and in our experience the method is innocuous. An apparatus utilizing such a procedure was devised and in numerous trials has proved quite satisfactory.

The equipment is relatively simple, consisting of a sterile apparatus, a frame, and a source of negative pressure.

The sterile apparatus (Fig. 2, A and C) consists of two L-shaped glass tubes with  $\frac{1}{4}$  by  $\frac{1}{4}$  inch legs (*F*) and  $2\frac{1}{4}$  by  $\frac{1}{4}$  inch legs (*X*), inserted into two openings of a three-hole No. 5 black rubber stopper (*R*), with the third opening containing a  $6\frac{1}{2}$  inch long glass tube (*A*) connected at its upper end by a  $1\frac{1}{2}$  inch flexible tube (*AB*) to a three-way metal stopcock (*SC*). A  $13\frac{1}{2}$  inch long flexible tube (*B*) connects the outlet of the stopcock with a nasal tip (*T-1*) by means of a  $3\frac{1}{2}$  inch glass tube (*C*). A similar nasal tip (*T-2*) and glass tube (*D*) is attached to a 9 inch long flexible tube (*E*) connecting with the end of the left lateral L-shaped tube (*F*). Nasal tips (*T-1*) have been made in three sizes from red rubber serum bottle stoppers by grinding down the sides.

All glass tubing is 7 mm. diameter Pyrex, annealed after bending. Flexible tubing may be of rubber or plastic but should be resistant to autoclaving and moderate negative pressure.

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In use, the set (wrapped as a unit and sterilized by autoclaving) is unwrapped and inserted by the rubber stopper (*R*) into a 100 ml. round-bottomed Pyrex centrifuge tube (or equivalent) (*CF*) containing 50 ml. of sterile nasal washing menstruum. A sterile 30 ml. syringe (preferably Luer Lock) (*S*) is attached to the stopcock.

The plywood frame (Fig. 2, *B*) for holding together and protecting the apparatus has a gun-shaped pattern. A  $\frac{3}{4}$  inch thick plywood block (*L*) with a  $1\frac{1}{8}$  inch diameter hemispheric depression and a rectangular opening (*M*)  $1\frac{1}{8}$  by  $5\frac{1}{2}$  inches support the centrifuge tube. A  $\frac{7}{8}$  by  $4\frac{3}{4}$  inch rectangular opening (*N*) holds the barrel of the 30 ml. syringe. Three small-

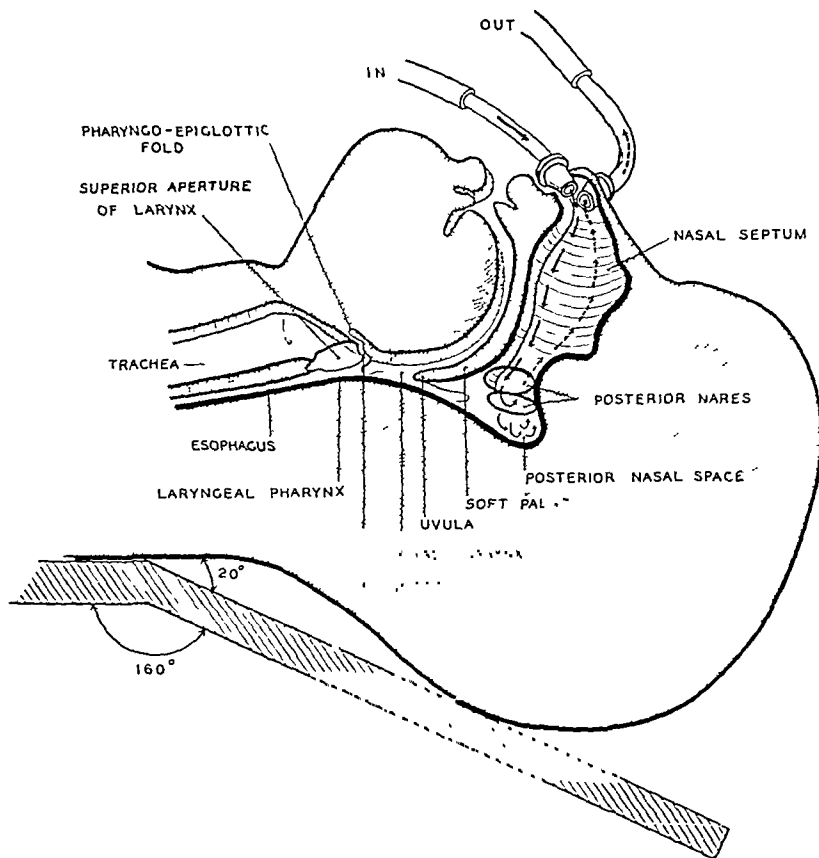
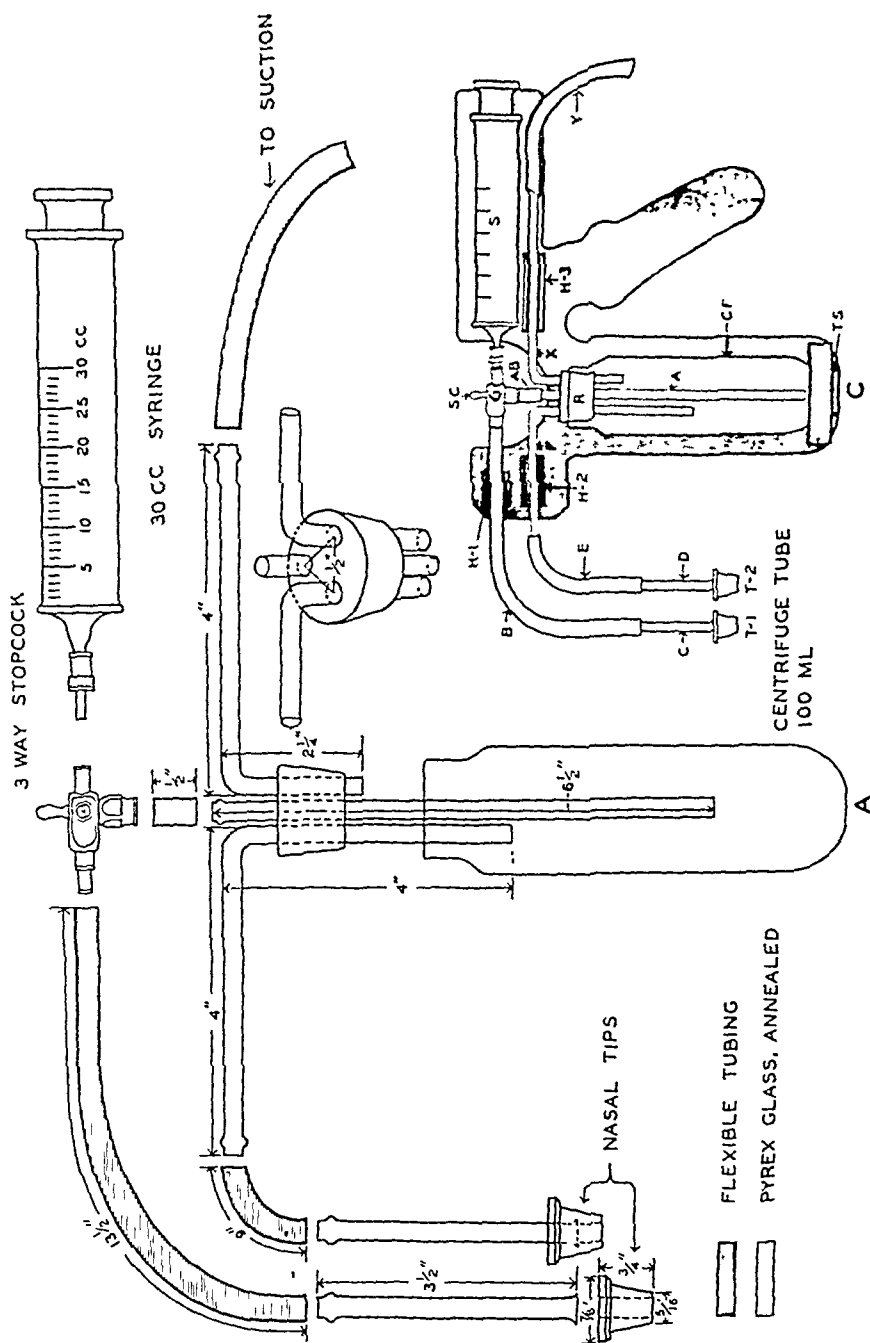


Fig. 1.

grooved pieces of wood (*H-1*, *II-2*, and *II-3*) support the tubing. They are secured to the frame by one screw apiece, which allows them to be turned to fit the tubing in the best possible manner. Attached to the base of the frame is a  $1\frac{1}{4}$  inch diameter by  $\frac{1}{4}$  inch thick threaded brass tripod socket (Fig. 2, *C*, *TS*). A sturdy camera tripod with telescoping legs and a good pan head is of value in supporting the apparatus at precisely the optimum position beside the donor's nose. Rubber bands conveniently secure the set to the frame, so that various-sized syringes and types of tubes containing nasal washing menstruum can be easily interchanged.





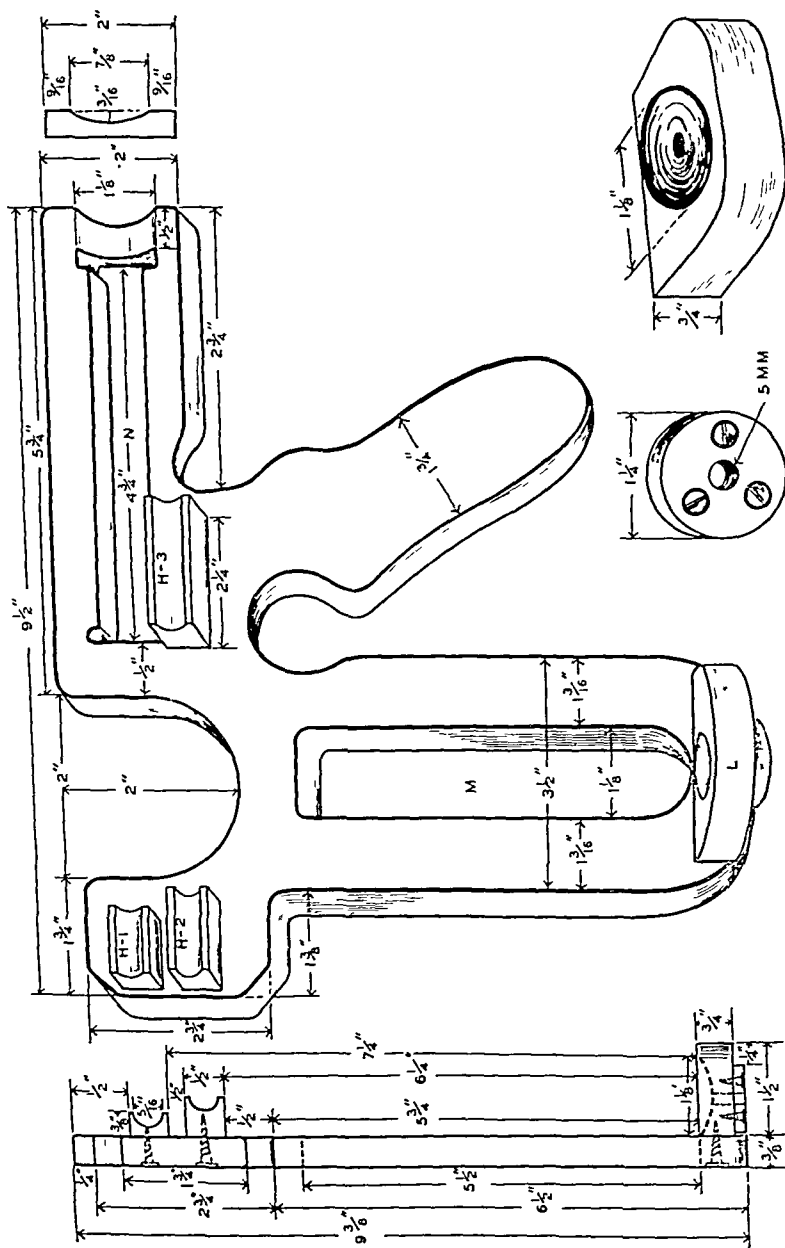


Fig. 2 (Cont'd).

A source of suction has been conveniently supplied by a water pump with adapters for various-sized water faucets. The optimum negative pressure is from 5 to 7 mm. of mercury.

#### TECHNIQUE OF OPERATION

The equipment is assembled as shown in Fig. 2, *C*. The sterile apparatus is unwrapped and the rubber stopper (*R*) inserted into a tube (*CF*) containing sterile nasal washing menstuum. A sterile 30 ml. syringe (*S*) is attached to the stopcock. Rubber bands are used to mount the assembly on the plywood frame.

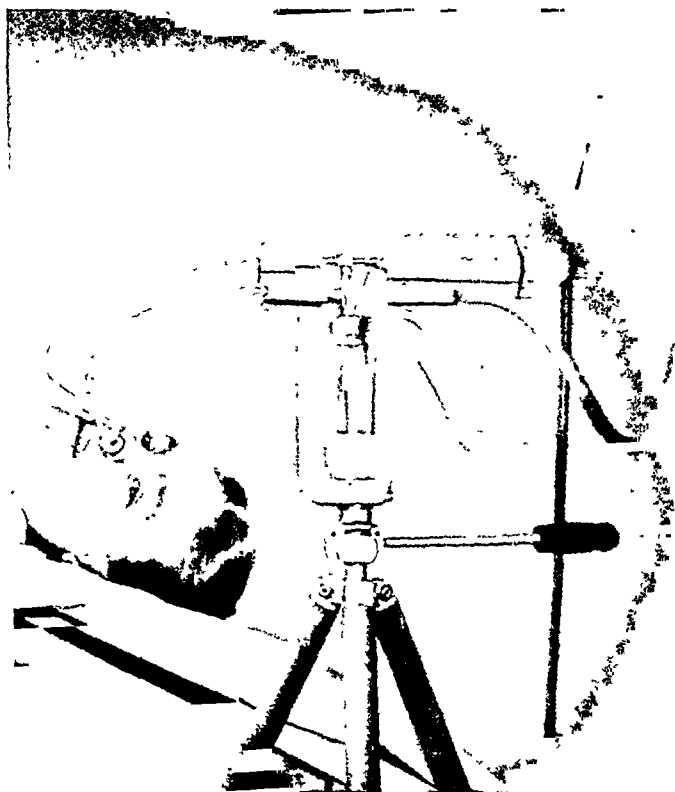


Fig. 3.

The suction tube (*Y*) is connected to the source of negative pressure. This is adequately tested by manually clamping tube *E* with the stopcock lever down (Fig. 4, *B*, position *a*). At the proper negative pressure, the 50 ml. menstuum begins to froth violently without, however, climbing into the L-shaped tube (*X*) connecting with suction.

The donor is requested to lie on the back with the head extended 20 degrees or more below the horizontal plane of the shoulders and body (Figs. 1 and 3). With the donor's head rotated to the side of the apparatus, nasal washing fluid can run out the side of the mouth into a sterile beaker should

the suction fail and the patient be unable to close off the posterior nasal recess by the soft palate and uvula. The nasal tips (Fig. 2, *C*, *T-1* and *T-2*) are gently but snugly inserted into the external nares and the donor instructed to breathe through the mouth. Usually the nasal tips must be kept in place by the operator, the donor, or an assistant.

Nasal washing is obtained in the following manner (Fig. 4):

1. With the stopcock lever up (Fig. 4, *A*, position *c*), the donor can usually feel the negative pressure. He is instructed not to swallow the menstuum.

2. With stopcock lever down (Fig. 4, *B*, position *a*), 30 ml. of nasal washing menstuum is drawn into the syringe.

3. Stopcock lever is turned to horizontal (Fig. 4, *C* and *D*, position *b*) and the material in the syringe is slowly emptied into the upper nostril, the posterior nasal recess of the nasopharynx, and through the lower nostril back into the apparatus (Fig. 1 and Fig. 4, *C* and *D*). When the syringe is free and the suction working well, the syringe will empty itself at the proper rate. Usually, however, the syringe must be manually operated. If the nasal washing menstuum is pushed through too rapidly (particularly if the suction is not adequate), fluid may overflow at the nasal tips, perhaps flow into the oral pharynx (where the patient may swallow it or expectorate it into a sterile beaker), or flow into the nasolacrimal duct to the eye.

Steps 2 and 3 are repeated as many times as desired. Utilizing this procedure, it is not difficult to circulate rather quickly 30 ml. amounts of menstuum through the nasal passages eight to ten times.

4. When the nasal passages have been sufficiently washed, the stopcock lever is turned up (Fig. 4, *E*, position *c*) so that the negative pressure cleans out both nostrils. The donor is instructed to inhale, sit up, and expectorate the material from the throat into the sterile beaker immediately after the nasal tips are removed. Usually, the expectorated material is only mucus showing no trace of the nasal washing menstuum. Often, however, the sinuses have been irrigated by the the washing procedure and a few milliliters more washing can be collected by having the donor flex the head and blow one side of the nose and then the other into the sterile beaker at intervals for two to five minutes.

The apparatus may be tipped slightly, if necessary, to capture the last few drops of mucus in the tubing. Suction is discontinued. The apparatus is disconnected from the tube containing the washing by pulling out the rubber stopper (Fig. 2, *C*, *R*). The material collected in the beaker may be poured into the tube if desired. The container (Fig. 2, *C*, *CF*) is closed by inserting a sterile No. 6 rubber stopper. Spraying with a vasoconstrictor will maintain the patient's comfort for a number of hours. This has not been necessary when sterile skim milk was used as the nasal washing menstuum.

Sterile skim milk has been the menstuum used for the routine nasal washings because many rickettsial agents survive longer and withstand freezing and drying better when protected by milk.<sup>1, 2</sup> Since a quantity of mucus has

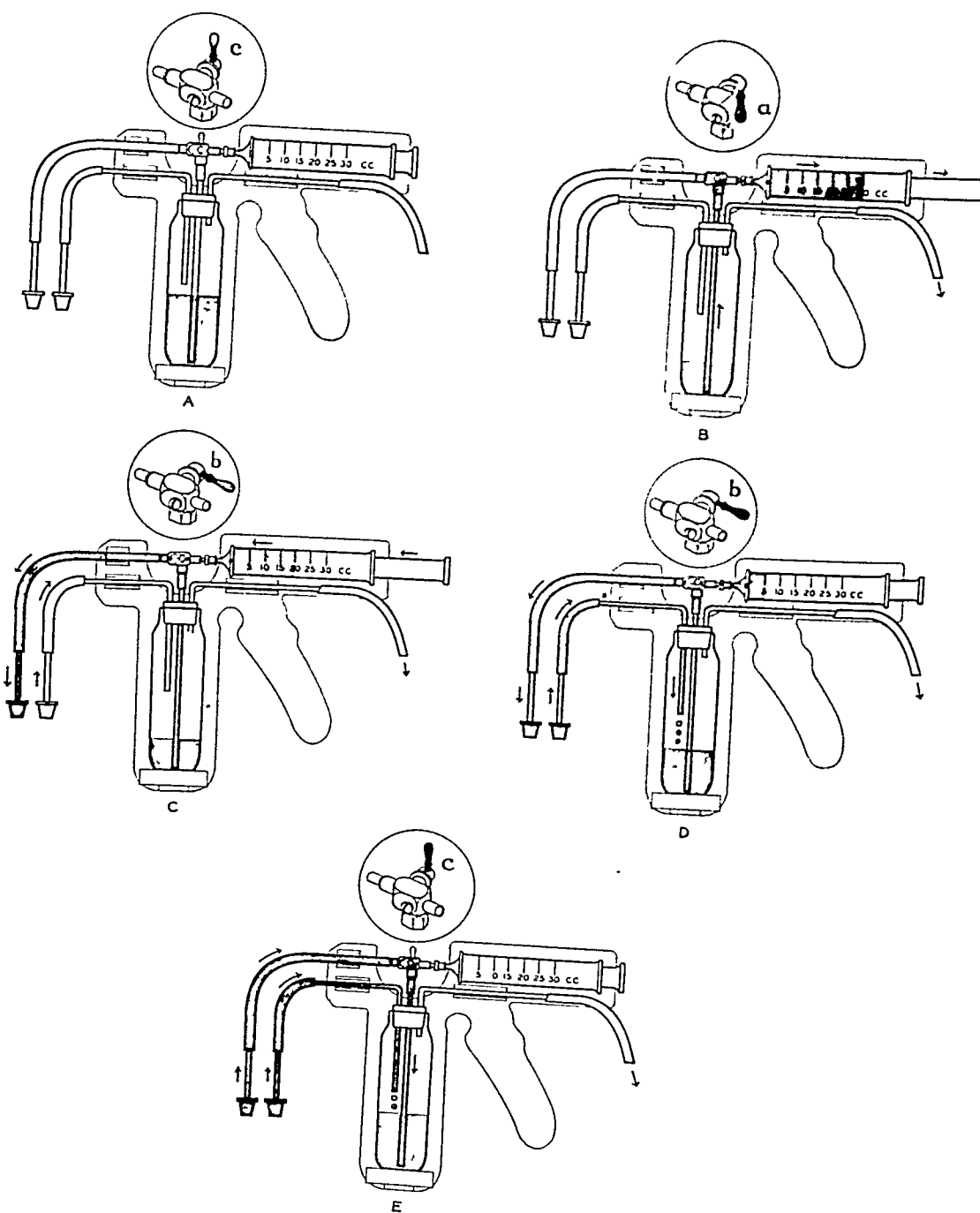


Fig. 4.

been present in most washings, homogenization in a Waring blender or by hand has been routine. After centrifuging (to remove cellular debris), quick freezing, and storing at  $-50^{\circ}$  C., washings are used for the inoculation of embryonated eggs, human volunteers, and various laboratory animals.

#### DISCUSSION AND SUMMARY

An apparatus and method for obtaining nasal washings is described. The apparatus and the method of its use have proved quite satisfactory during numerous trials. No complications have resulted from obtaining nasal washings in this manner.

Advantages of such a procedure are as follows:

1. It obtains washings from the nasal passages without serious contamination from oral material.
2. Washings reach areas in the nasal passages (such as sinuses and nasolacrimal ducts) not usually reached by previous methods.
3. Friable epithelial cells and cellular debris in nasal passages are collected more effectively by recirculating the washing material eight to ten times. The etiologic agents sought may be present in this cellular portion.
4. Washings are easily and efficiently collected.
5. Procedure is not as unpleasant as previous methods, and donors report a feeling of well-being and "clearheadedness" following the washing.

#### REFERENCES

1. Topping, N. H.: The Preservation of the Infectious Agents of Some of the Rickettsioses, *Pub. Health Rep.* 55: 545, 1940.
2. Anderson, C. R.: Survival of *Rickettsia prowazeki* in Different Diluents, *J. Bact.* 47: 519, 1944.

## A NEW RAPID TECHNIQUE FOR Rh TYPING

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IT IS being recognized increasingly that human isoimmunization to the Rh factors is a common occurrence. Whereas about only one in twenty-five Rh-negative women who bear Rh-positive infants ever develop Rh antibodies, Diamond<sup>1</sup> and Wiener and Sonn-Gordon<sup>2</sup> were able to sensitize a very large percentage of Rh-negative volunteers by injections of Rh-positive blood. Diamond,<sup>1</sup> Ross,<sup>4</sup> and Hattersley<sup>3</sup> likewise found Rh antibodies in the serum of about 50 per cent of previously transfused Rh-negative patients, demonstrating that sensitization by transfusion is not, as it has been considered for so long, a relatively rare occurrence. The danger to future pregnancies of such accidental sensitization in women is well established, and the fact is becoming recognized that transfusion reactions due to Rh sensitization occur not infrequently. Consequently, increasing numbers of hospital laboratories are establishing Rh typing as a routine pretransfusion procedure.

There are three important difficulties which are encountered when such a program is introduced: (1) Adequate supplies of reliable and potent Rh typing serum are not always available; (2) Rh typing techniques require skill which many laboratory technicians lack; (3) Rh typing requires time which, when blood is urgently needed, is at a premium.

The first of these difficulties is now largely solved by the increasing use of the hyperimmune or "blocking" antibody, which was first shown by Diamond and Abelson<sup>5</sup> to be capable, under certain conditions, of agglutinating Rh-positive cells. Sera containing such antibodies are of common occurrence, particularly among individuals sensitized by transfusions, and may reach extremely high titers. They have the further advantage of being more stable than saline agglutinins and, with the techniques suggested by Wiener<sup>6</sup> and Diamond and Denton,<sup>7</sup> are equally easy to use.

On the other hand no method of Rh typing which is at once uniformly easy to read and very rapid has yet been reported. The tube techniques, while perhaps the simplest to interpret, have the drawback of requiring incubation. The slide technique of Diamond and Abelson,<sup>5</sup> while it gives Rh typing in five minutes or less, occasionally gives confusing or equivocal results, and the somewhat less rapid capillary method of Chown<sup>8</sup> requires a skill of handling which makes it impractical for many smaller laboratories.

While attempting to solve these problems as they existed in this laboratory, it was discovered that when Rh-positive cells are mixed with a high titer hyper-

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immune Rh antiserum and immediately centrifuged at high speed, there is gross agglutination which is not readily broken up. On the basis of this observation, the following technique was established:

A potent hyperimmune serum (with alpha and beta antibodies neutralized) is diluted with several volumes of 20 to 30 per cent bovine albumin. One drop of this mixture is placed in a small agglutination tube. An applicator stick is thrust into the clotted or oxalated whole blood to be tested and enough cells carried over to the agglutination tube to make a moderately heavy suspension in the drop of serum.\* (If preferred, a small drop of a suspension of cells in their own serum or in saline may be used, taking care that the concentration of albumin in the final mixture does not fall below 15 per cent.) The tube



Fig. 1.—The Rh test, ready to read. Tube on the left shows gross agglutination of Rh-positive cells. Tube on right shows smooth suspension of Rh-negative cells. (Courtesy Letterman General Hospital Photographic Laboratory.)

is shaken to insure mixing and without preliminary incubation is centrifuged at high speed for two minutes. It is then shaken gently and the sediment examined for agglutination. Rh-positive cells show gross clumping which is not readily broken up. Rh-negative cells remain perfectly smooth, without rouleaux (Fig. 1).

Since this technique was introduced into our laboratory, where it has been used in parallel with one of the standard methods, it has proved entirely reliable in more than 1,000 tests. It has been found so easy to handle that even our least skilled technicians have had no trouble with it, and its great speed has on several occasions proved invaluable to us.

Certain precautions are necessary in choosing a suitable serum for use with this technique. Sera with an initial titer in albumin of less than 1,000 are

\*This portion of the technique was suggested by Miss Evelyn Tennis of the Community Blood Bank, San Mateo, Calif.

seldom satisfactory, and the final titer should not be below 100. Even among sera of adequate titer some will be found which are insufficiently avid to give satisfactory results, and these sera must be eliminated by trial and error. Care should likewise be taken to standardize the serum as to specificity, for, as shown by Diamond<sup>1</sup> and Hattersley,<sup>4</sup> hyperimmune antibodies of Rh' (C of Fisher) and Rh'' (E) specificity not infrequently occur, mixed with the more common Rh<sub>0</sub> (D) antibody.

It is felt that if these few precautions are observed this rapid technique should prove of considerable value, particularly in laboratories such as ours where speed and ease of Rh typing are at a premium, and should render it increasingly practical to determine routinely the Rh type of all transfusion cases.

#### SUMMARY

A new technique of Rh typing is described. It utilizes the readily available Rh "blocking" serum and has the advantage of speed and of ease of reading. It has proved entirely reliable in a series of over 1,000 bloods.

#### REFERENCES

1. Diamond, L. K.: Physicochemical and Immunological Characteristics of Rh Antibodies; read at the International Hematology and Rh Conference, Dallas, Texas, Nov. 16, 1946.
2. Wiener, A. S., and Sonn-Gordon, E. B.: Simple Method of Preparing Anti-Rh Serum in Normal Male Donors, *Am. J. Clin. Path.* 17: 67, 1947.
3. Ross, J. B.: Personal communication.
4. Hattersley, Paul G.: Two Popular Fallacies Regarding Rh—Preliminary Report of Some Thought Provoking Observations, *J. LAB. & CLIN. MED.* 32: 423, 1947.
5. Diamond, L. K., and Abelson, N. M.: The Demonstration of Anti-Rh Agglutinins—An Accurate and Rapid Slide Test, *J. LAB. & CLIN. MED.* 30: 204, 1945.
6. Wiener, A. S.: Conglutination Test for Rh Sensitization, *J. LAB. & CLIN. MED.* 30: 662, 1945.
7. Diamond, L. K., and Denton, R. L.: Rh Agglutination in Various Media, With Particular Reference to the Value of Albumen, *J. LAB. & CLIN. MED.* 30: 821, 1945.
8. Chown, B.: Rapid, Simple, and Economical Method for Rh Agglutination, *Am. J. Clin. Path. Tech. Sect.* 8: 114, 1944.



tivity of measurement as determined with the Coleman Junior Spectrophotometer is greatest at 540 millimicrons. A filter with the maximum transmittance at 540  $m\mu$  was used in the routine determinations with the Evelyn colorimeter.

*Recovery Experiments.*—Serum was obtained from various species by allowing freshly drawn blood to clot and storing it in the refrigerator for a few hours. Each sample of serum was analyzed for magnesium according to the technique described. Known amounts (10 to 20  $\mu\text{g}$  per milliliter) of magnesium, as magnesium sulfate, were then added to the serum and analyses were run to measure the recovery of the added magnesium. Data were obtained from twenty-four determinations on twelve different serum samples. The mean recovery of the added magnesium was 99.84 per cent, with a standard deviation of  $\pm 1.44$  per cent. Whole blood was oxalated by means of potassium or sodium oxalate. The procedure used in the recovery experiments was the same as that used with serum, except that 20 to 40  $\mu\text{g}$  of magnesium were added. The mean recovery of thirty-seven determinations on twelve different blood samples from different species was 99.47 per cent, with a standard deviation of  $\pm 1.32$  per cent.

Urine obtained from male adults was used in the recovery experiments. The mean recovery for ten determinations of added magnesium to ten samples was 99.65 per cent, with a standard deviation of  $\pm 2.22$  per cent.

TABLE I. RECOVERY OF MAGNESIUM ADDED TO SAMPLES OF SERUM, WHOLE BLOOD, AND URINE

	SERUM	WHOLE BLOOD	URINE
Number of samples	12	12	10
Number of determinations	24	37	10
Mean per cent magnesium recovered	99.84	99.47	99.65
Standard deviation	$\pm 1.44$	$\pm 1.32$	$\pm 2.22$

In all recovery experiments there was no constant error in the method since the per cent recovery was neither consistently above nor below the expected value.

Ten samples of serum were independently analyzed by the described method on different days by two different analysts using two entirely different sets of reagents. The correlation coefficient between the magnesium values obtained by the two different individuals was  $+0.985$ . Also in analyses of ten samples of whole blood, different amounts of filtrate (3 to 5 ml.) were used and a correlation coefficient of  $+0.961$  was obtained. There was no constant error, for the values obtained using smaller aliquots were neither consistently above nor below the values obtained using larger aliquots.

*Remarks.*—Calcium and phosphate ions interfere, but fortunately these substances have not been found to be present as blood components in concentrations that appreciably influence the results. The calcium concentration, as reported by Gillam,<sup>4</sup> in order to interfere must be at least 500  $\mu\text{g}$  per milliliter in the solution that is read photometrically. The phosphorous concentration must be kept below 100  $\mu\text{g}$  per milliliter since it interferes by buffering the solution and lowering the hydroxyl ion concentration. If in urine, for

example the urine of a calf receiving only a milk diet for some time, the phosphorus-magnesium ratio is wider than 200:1, the method described in this paper is limited to a semiquantitative determination. If such urine is to be analyzed, the technique for soil extracts described by Gillam<sup>4</sup> is recommended.

The hydroxyl ion concentration must be carefully controlled. The higher the hydroxyl ion concentration, the greater the density of the solution. Acid, base, or buffering solutions (hydroxylamine hydrochloride solution) must be added carefully so as to insure the same amounts in the unknown sample as in the blank. In the method described by Garner<sup>7</sup> the alkalinity of the unknown was greater than that of the blank, for the method did not take into account the trichloroacetic acid removed from the solution by the precipitation of the proteins. The values were higher than those obtained when the trichloroacetic acid had been removed.

The temperature of the solution affects the optical density of the solution and must be controlled. The specific temperature is not of importance but the unknown sample should be read at approximately the same temperature as the standards. It is more convenient to adjust to room temperature; therefore the temperature selected was 25° C.

TABLE II. THE MAGNESIUM CONTENT OF WHOLE BLOOD AND PLASMA

SPECIES	WHOLE BLOOD			PLASMA OR SERUM		
	NUMBER OF ANIMALS	AVERAGE (MG./100 ML.)	RANGE* (MG./100 ML.)	NUMBER OF ANIMALS	AVERAGE (MG./100 ML.)	RANGE* (MG./100 ML.)
Human being	6	3.82	3.58-4.50	6	2.14	2.02-2.22
Sheep	6	2.79	2.61-2.92	6	2.27	2.02-2.47
Rabbit	7	5.23	4.72-5.73	6	2.45	2.05-2.97
Pig	6	5.26	5.03-5.60	8†	2.46	2.20-2.84
Cattle	6	2.04	1.95-2.09	45†	2.13	1.23-2.75

\*Range refers to observed concentrations.

†Serum.

*Magnesium Content of Blood.*—The magnesium content of whole blood and plasma or serum from five different species has been determined by the method reported here. The bloods were collected at random from healthy individuals and animals. The results (Table II) show that the concentration of magnesium in the plasma or serum varies little among the different species. There was considerable variation in the magnesium content of whole blood. The magnesium content of the whole blood of the rabbit and pig was around 5.24 mg. per 100 ml., whereas for cattle and sheep the values were less than 3.0 mg. per 100 milliliters. In the human being, sheep, rabbit, and pig the concentration of magnesium in the whole blood is greater than that in the plasma or cells. These observations are in accord with reports on the distribution of magnesium in the blood of cattle<sup>8</sup> and human beings.<sup>9</sup>

#### SUMMARY

A method has been described for the photoelectric or spectrophotometric determination of the magnesium content of serum, plasma, whole blood, erythro-

cytes, and urine. The blood proteins are precipitated by means of tungstic acid, and the magnesium is measured by the amount of the red magnesium hydroxide-titan yellow complex produced. The mean recoveries of added magnesium ranged from 99.5 to 99.8 per cent with whole blood, serum, and urine.

Data are presented on the magnesium content of whole blood and plasma for the human being, rabbit, pig, sheep, and cattle.

#### REFERENCES

1. Power, M. H.: Colorimetric Determination of Magnesium, *Federation Proc.* 1: 130, 1942.
2. Briggs, A. P.: A Colorimetric Method for the Determination of Small Amounts of Magnesium, *J. Biol. Chem.* 52: 349, 1922.
3. Kolthoff, I. M.: Eine neue spezifische Farbreaktion auf Magnesium und eine einfache kolorimetrische Methode zur quantitativen Bestimmung von Spuren dieses Elements, *Biochem. Ztschr.* 185: 344, 1927.
4. Gillam, W. S.: A Photometric Method for the Determination of Magnesium, *Indust. & Engin. Chem. (Anal. Ed.)* 13: 499, 1941.
5. Lindner, R. C.: Rapid Analytical Methods for Some of the More Common Inorganic Constituents of Plant Tissues, *Plant Physiol.* 19: 76, 1944.
6. Ludwig, E. E., and Johnson, C. R.: Spectrophotometric Determination of Magnesium by Titan Yellow, *Indust. & Engin. Chem. (Anal. Ed.)* 14: 895, 1942.
7. Garner, R. J.: Colorimetric Determination of Magnesium in Plasma or Serum by Means of Titan Yellow, *Biochem. J.* 40: 828, 1946.
8. Eveleth, D. F.: Comparison of the Distribution of Magnesium in Blood Cells and Plasma of Animals, *J. Biol. Chem.* 119: 289, 1937.
9. Greenberg, D. M., Lucia, C. P., Mackey, M. A., and Tufts, E. V.: The Magnesium Content of the Plasma and Red Corpuscles in Human Blood, *J. Biol. Chem.* 100: 139, 1933.

# RAPID TITRIMETRIC MICROMETHOD FOR THE DETERMINATION OF NONPROTEIN NITROGEN

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PETAH TIQA, PALESTINE

IT IS possible to determine the nonprotein nitrogen in whole blood, serum, or plasma within a few minutes with the accuracy of the Kjeldahl method<sup>1</sup> but without the troublesome digestion and distillation. An alkaline hypobromite-boric solution is added to a protein-free filtrate and the excess of bromine is titrated iodometrically.<sup>2, 3</sup> Boric acid has been added to the deaminating solution to prevent any interaction between the bromine and the glucose present in protein-free filtrates, since even large amounts of glucose are bound by boric acid. Not only urea and ammonia, but all physiologic amino compounds are included, the latter being deaminated simultaneously. Even amino acid mixtures obtained by protein digestion with trypsin in the preparation of broth give identical results using this and the Kjeldahl method. Indole compounds which contain nitrogen in their ring system do not affect the result since normal blood contains only traces of these compounds.<sup>4</sup> Even in uremia, indole compounds do not interfere since their nitrogen contribution does not rise above 0.4 to 0.5 mg. per cent.

## METHOD

### *Reagents.—*

#### *A. Deproteinizing Solutions:*

1. Dissolve 20 Gm. sodium sulfate anhydrous in water, add 69 ml. of  $\frac{2}{3}$  N sulfuric acid and fill the flask with water to the 1,000 ml. mark.

2. Dissolve 5 Gm. sodium sulfate and 8.3 Gm. phosphomolybdic acid in about 200 ml. water; add 20 ml. of 5 N sodium hydroxide solution and boil gently over a burner for thirty minutes. Cool; add 10.6 ml. of concentrated sulfuric acid, then 10 Gm. cadmium sulfate, and make up to final volume of 1,000 milliliters.

Cadmium sulfate has been added to the deproteinizing solution in order to precipitate compounds which would evolve hydrogen sulfide and which would therefore use up relatively large amounts of bromine. However, cadmium sulfate precipitates all sulfur compounds.

#### *B. Preparation of Deaminating Reagent:*

1. Boric acid solution. Dissolve 84.5 Gm. boric acid and 15.6 Gm. sodium hydroxide in about 500 ml. water and boil for thirty minutes. Cool and make up to one liter.

2. Sodium fluoride solution saturated.

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3. Sodium hydroxide, 27 per cent. Made by dissolving 27 Gm. in 100 ml. of water.

4. Bromine solution. Dissolve 20 Gm. potassium bromide in about 50 ml. water; add 8 Gm. (2.5 ml.) pure bromine; mix until bromine dissolves and make up to one liter.

If it is more convenient, the bromine solution may be made as follows: Dissolve 32 Gm. potassium bromide and 2.8 Gm. potassium bromate ( $\text{KBrO}_3$ ) in some water in a liter volumetric flask; add 100 ml. of normal sulfuric acid and after waiting for thirty minutes, fill up to the 1,000 ml. mark with water.

5. Boric acid-fluoride mixture. Mix 5 parts boric acid solution (1) with 3 parts sodium fluoride (2) and 1 part 27 per cent sodium hydroxide (3) [for example, to 250 ml. (1) add 150 ml. (2) and 50 ml. (3)]. This mixture keeps well.

6. Deaminating reagent. Just before use add 1 part of bromine solution to 9 parts of boric acid-fluoride mixture.

#### C. Titration Reagents:

1. Sodium thiosulfate N/200.
2. Potassium iodide crystals.
3. Starch solution 0.25 per cent.
4. Hydrochloric acid 18 per cent (Approximately 5 N).

*Procedure.*—Pipette 2.9 ml. deproteinizing solution (A1) into short, stoppered test tubes. With a capillary pipette add 0.1 ml. blood directly from finger and lake by repeatedly drawing fluid in and out of the pipette. Add 1 ml. of deproteinizing solution (A2). Mix, let stand five minutes, and then centrifuge or filter (0.2 ml. blood may be used with 5.8 ml. deproteinizing solution, and 2 ml. of phosphomolybdic acid).

Into two wide test tubes or flasks, pipette 3 ml. of clear filtrate and 5 ml. of deaminating solution. Mix and, after two minutes, add a few crystals of potassium iodide and 2 to 3 ml. of hydrochloric acid to each sample and titrate with N/200 sodium thiosulfate until yellow; finally, add a few drops of starch solution and titrate until disappearance of the blue color.

As a blank, mix 3 ml. of deproteinizing solution (1) with 1 ml. of deproteinizing solution (2); take 3 ml. of the mixture to add to 5 ml. of deaminating solution and titrate with the thiosulfate solution.

*Calculation.*—Since  $2\text{NH}_3 + 6\text{Br} = 6\text{HBr} + \text{N}_2$ , one atom of N is equivalent to 3 atoms Br. Thus 1 ml. N/200 thiosulfate is equivalent to

$$\frac{14 \text{ (atomic weight N)}}{3 \times 200} = 0.0233 \text{ mg. } \frac{160}{0.075 \text{ (ml. sample used)}} \times 0.0233 = 31.1.$$

From the milliliters of thiosulfate used for the blood sample, subtract the milliliters used for the reagent blank, and obtain the milliliters actually used by the blood sample. Multiply this by the factor 31.1 to obtain the nitrogen in milligrams per cent.

## ALTERNATE METHOD

*Reagents.*—*A. Deproteinizing Solution* (Modified Abrahamson's Reagent)<sup>2</sup>:

Dissolve 4.48 Gm. sodium tungstate, 2 Gm. sodium citrate and 6.4 Gm. sodium sulfate in about 500 ml. water; add 44.8 ml. of a normal sulfuric acid or 67.2 ml. 2/3 N sulfuric acid; then add 2 Gm. cadmium sulfate and fill volumetric flask to 1,000 milliliters.

*Procedure.*—Pipette 5.0 ml. deproteinizing solution A into a short test tube. Wash in 0.1 ml. finger-tip blood from capillary pipette. After five minutes, centrifuge or filter. Place 4 ml. of clear filtrate in another tube and continue with deamination and with the thiosulfate titration as described. As a blank, use 4 ml. of deproteinizing solution and 5 ml. of deaminating solution.

*Calculation.*—Subtract the number of milliliters of N/200 sodium thiosulfate used in the blank determination from the amount used by the blood sample, and multiply by 29.75 as a factor to obtain the amount of nitrogen in milligrams per cent, since 0.0784 ml. of blood is the amount of blood used in the determination.

## SUMMARY

A rapid, accurate, and convenient titrimetric procedure has been described for the estimation of nonprotein nitrogen in serum, plasma, or whole blood. Since this method does not require digestion and distillation of the sample, many errors are avoided which are inherent in the longer procedure.

TABLE I. COMPARISON OF THE PRESENT METHOD WITH THE KJELDAHL METHOD FOR DETERMINATION OF NONPROTEIN NITROGEN

NUMBER	MICRO-KJELDAHL METHOD <sup>1</sup> (MG. PER 100 ML.)	PRESENT METHOD (MG. PER 100 ML.)
1	199	199
2	213	215
3	31.1	32.6
4	31.1	29.5
5	15.5	14.5
6	23.3	23.3
7	18.6	17.1
8	44.0	43.5

## REFERENCES

1. Pregl, F.: Quantitative Organic Microanalysis, Philadelphia, 1924, P. Blakiston's Son & Co.
2. Rappaport, F.: Microdetermination of Residual Nitrogen and Urea in the Blood, *Mikrochemie* 14: 48, 1933.
3. Rappaport, F.: Improvement of the Titrimetric Method of Nonprotein Nitrogen in Blood Without Distillation, *Klin. Wehnschr.* 16: 1190, 1937.
4. Rappaport, F.: Determination of Nonprotein Nitrogen and Its Fractions. III. A Micro-method for Determining Indican in the Blood, *Klin. Wehnschr.* 12: 1184, 1933.
5. Welcher, F. J.: Chemical Solutions, New York, 1942, D. Van Nostrand Company, Inc.

## FIBRIN APPEARANCE TIME

### A ROTATING TUBE METHOD FOR ESTIMATING THE CLOTTING TIME OF THE BLOOD

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THERE is great need for a test to reflect more reliably the fine alterations in the over-all picture of the coagulability of the blood than is possible by current methods. The results are influenced especially by (1) the constituents of the blood, (2) crude technical manipulation, and (3) uncertain criteria for fixing the time of clotting, the end point. Items 2 and 3 are amenable to control, and a technique which would make them more constant or precise would yield a more critical test than those in general use.

Considerable variation in the content, or activity, of one or more factors in the clotting process may occur without demonstrable alteration in the coagulation time, as it is commonly determined. This is well demonstrated in dicumarol- or salicylate-induced hypoprothrombinemia. It is possible, however, that a more sensitive test would reflect regularly the inhibition of the clotting process in the presence of reduced activity of a single component such as prothrombin.

In the manipulation of the blood specimen there are two major factors which alter the coagulation time: (1) the nature of the container and (2) trauma to the formed elements of the blood.

*Nature of the Container.*—When kept in “nonwetable” vessels, blood may clot more slowly. This can be controlled by using the same type of vessels of approximately equal size and smoothness at all times. Inconstancy in the relationship of glass contact surface to total volume of blood will give considerable alteration in clotting time. Thus, by the Howell technique, using 21 mm. diameter tubes, the clotting time of normal blood may extend to sixty minutes; by the Lee-White method, using tubes of 8 mm. in diameter, the normal range for blood coagulation is from four to sixteen minutes; by the capillary tube methods, with tubing of less than 1 mm. in diameter, the normal clotting time is likewise highly variable. Nor can blood be poured indiscriminately against the glass surfaces of tubes without affecting adversely the reliability of the test. This can be demonstrated by comparing the Lee-White clotting time of a slightly tilted and of a fully tilted set of tubes. We have observed that not only the frequency and angle of tilting, but also the constancy of direction of tilting make a difference in the clotting time.

*Trauma to Blood Constituents.*—Blood which is forced under pressure through a small aperture, or is shaken or stirred, will clot promptly due to trauma to the formed elements, especially platelets, and to the liberation of the coagulating factors into the blood sample. To obviate this, it is essential that the blood be transferred consistently and gently.

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*Lee-White Coagulation Test.*—If a tube containing blood is tilted at regular intervals, the viscosity of the blood increases gradually until it forms a gelatinous mass which projects above the liquid portion when the position of the tube is changed. Later, the blood solidifies except for a small amount of liquid at the rim. Eventually, when the whole mass becomes solid enough to permit inversion of the tube without spilling, the end point is reached. Frequently the end point remains in doubt for several minutes, adding to the crudeness of the procedure. This difficulty is magnified in cases of disturbed clotting found in heparin medication and in blood dyscrasias.

#### FIBRIN APPEARANCE TIME

The following procedure minimizes the variables noted previously by providing (1) a nontraumatic manipulation, (2) constant agitation, and (3) a precise end point.

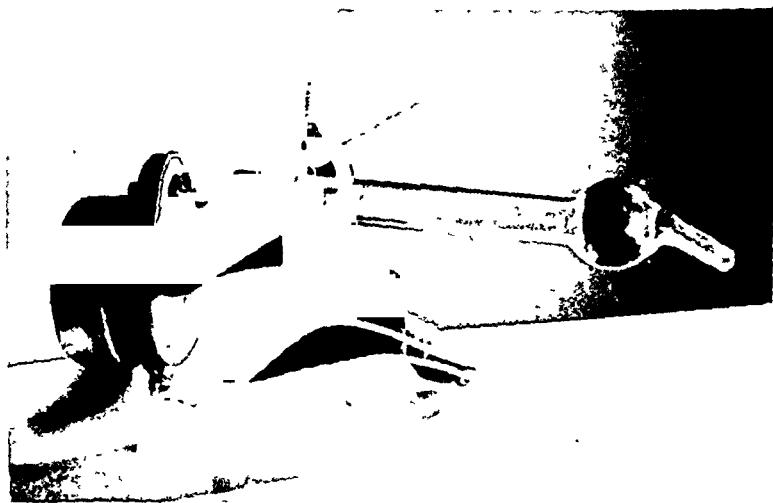


Fig. 1.—Photograph of apparatus showing tube attached to motor (see text).

In its liquid state, a small quantity of unmodified blood will remain, by virtue of its cohesive force, in a concave smooth container in the form of a large drop and will not attach itself to the surrounding surface. The instant blood commences to clot, fibrin forms and, because of its adhesiveness, clings to the container wall. This change is immediately detectable if the blood is contained in a transparent tube rotating at a slow and constant rate. Then, when fibrin forms it attaches to and climbs with the rising wall.

#### *Apparatus (Fig. 1).*—

1. A transparent tube of glass or plastic material about 11 mm. in diameter with a bulbous dilatation at one end from which there extends an eccentrically directed opening for the introduction of the blood sample.
2. A motor which rotates at a constant slow rate of about 1 revolution per minute.



3. An attachment to the shaft of the motor to hold the tube (Fig. 1).

*Procedure.*—A sample of blood is obtained by smooth venipuncture. At the instant of venipuncture the stop watch is started. One-half of one milliliter, about 8 drops, is immediately introduced into the bulbous end of the tube, which is attached to the shaft of the motor, and the motor is started. The blood forms into a large drop which remains in the well of the bulb. As soon as fibrin forms, the strands adhere in a growing mass to the surrounding bulb and climb with the rising wall. This appearance of a continuous mass of fibrin is the end point.

In one hundred estimations, made in eighty normal individuals, in which the rotating tube was subjected to the heat of a strong electric light, the time of fibrin appearance varied between 3.5 and 5 minutes. In thirty-one tests made in an equal number of cases at room temperature there was an average deviation of 33 seconds from a mean of 5 minutes and 18 seconds. We accept 4 to 6 minutes as the normal range at room temperature. In all normal cases in which blood was drawn gently after smooth venipuncture, the clotting time fell within the above range.\*

A comparison was made of the clotting time of blood by the Lee-White method and the rotating tube method. Specimens of blood were obtained by "clean" venipuncture on twelve patients with a variety of chronic diseases. Each sample was divided instantly, appropriate quantities being used for the respective tests. The average results obtained are shown in Table I.

TABLE I

	AVERAGE TIME (MIN.:SEC.)	AVERAGE DEVIATION	% DEVIATION FROM AVERAGE
		FROM MEAN (MIN.:SEC.)	
Rotating tube	5:15	0:39	12
Lee-White	10:38	2:36	24

From these data it can be seen that while the average clotting time by the Lee-White method is only twice as long as that by the rotating tube, the average deviation is four times as great.

The figures obtained are shown in Table II.

TABLE II

	ROTATING	LEE-WHITE
1	5:00	14
2	6:20	14
3	5:50	13
4	5:20	12½
5	6:15	8½
6	4:45	11
7	4:05	5½
8	4:10	8
9	5:25	7
10	5:00	8½
11	6:10	13
12	4:45	12.5

\*If it is desired to make the test at body temperature, this can be accomplished by placing a strong electric bulb near the apparatus. The distance must be determined by trial and the apparatus kept at the optimum point for a time interval adequate to insure the desired temperature.

Patients being treated with dicumarol present good correlation between prolonged prothrombin time and fibrin appearance in the rotating tube (Fig. 2). Fibrin appearance time will not replace prothrombin time estimations in following dicumarol therapy, but it may extend the interval between essential prothrombin time estimations to about every five days. Fibrin appearance time is taken daily at the bedside. It has been found to become prolonged to about ten minutes at the dicumarol therapeutic level. It restores to normal more promptly than the prothrombin time. As soon as the fibrin appearance time becomes normal the prothrombin estimation is made again, and further dosage of dicumarol is determined from these prothrombin time values.

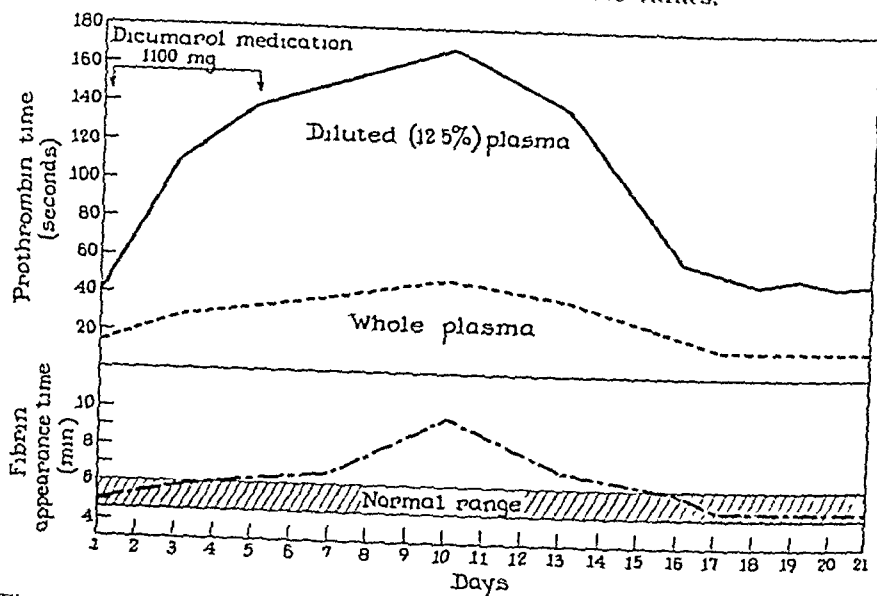


Fig. 2.—Chart illustrating extended fibrin appearance time in relation to prolonged prothrombin time of whole and diluted (12.5 per cent) plasma after 1,100 mg. of dicumarol.

The fibrin appearance method has a high correlation in heparin therapy as well. While the end point in the Lee-White method is vague and uncertain after heparin, the fibrin, appearance time method reveals a clear and unmistakable end point. We have found a good therapeutic level after heparin to be about twelve minutes.

In blood dyscrasias, characterized by extended coagulation time, the end points are likewise clear and the results reproducible. Thus we have found the fibrin appearance time to be 35 minutes in hemophilia. In thrombocytopenic purpura the time has been definitely, although only slightly, prolonged during the active bleeding phase of the disease. In the presence of clinical thrombosis in the acute stage the fibrin appearance time has been found to be normal or reduced.

#### SUMMARY

A method of precisely determining the over-all coagulability of unmodified blood by estimation of the time required for the appearance of fibrin is described. The test is made in a transparent tube rotating at a constant and slow rate.

It registers small increases in coagulation time, thus permitting a more critical estimation of alteration in blood coagulability.

It is applicable to the determination of the maintenance of therapeutic levels of heparin and/or dicumarol. Although it does not eliminate entirely the need for prothrombin time estimations after dicumarol, it may prolong considerably the intervals between essential prothrombin time estimations.

It may be possible to demonstrate acceleration of coagulation time by the new procedure.

## A RAPID TEST FOR ALBUMIN AND SUGAR IN THE SAME MEASURED SAMPLE OF URINE

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TESTS to determine the presence of albumin and sugar in urine are ordinarily made on two different samples of the specimen. It would be advantageous to be able to make these two tests on the same measured sample of urine. The procedure described permits this to be done. Sulfosalicylic acid is the reagent used for albumin, and Benedict's solution, for sugar.

### METHOD

Deliver 8 drops of urine into a test tube having an outside diameter of 19 millimeters. Next add 0.8 ml. of a 4.5 per cent solution of sulfosalicylic acid. The almost immediate appearance of a white, turbid precipitate or cloud indicates the presence of albumin. Now add 5 ml. of Benedict's solution and shake the tube well to mix the contents. Proceed as usual by boiling over the free flame for one or two minutes or by heating in a boiling water bath for five minutes. Allow the tube to cool in the air spontaneously. Interpret the result in the customary manner. This may be called the laboratory method.

The following modification should be useful to physicians, insurance medical examiners, visiting nurses, and others who do not wish to bother with reagent solutions. The test may be performed in the home, if desired. Deliver 5 drops of urine into a test tube having an outside diameter of 15 mm. and then add 10 drops of water. Drop in one sulfosalicylic acid reagent granule\* and shake the contents gently for a second or two. After two minutes look for the turbidity described in the preceding paragraph. The granule does not dissolve, but it carries sufficient sulfosalicylic acid which is released to precipitate the protein. Proceed with the test for sugar by dropping in one effervescent sugar reagent tablet,† the active ingredients of which are cupric sulfate, sodium hydroxide, sodium bicarbonate, and citric acid. About fifteen seconds after the reaction has ceased, a typical copper reduction test will be obtained in the presence of sugar.

### DISCUSSION

Sulfosalicylic acid is recognized as an excellent reagent for the precipitation of albumin in urine. The quantitative method described by Kingsbury, Clark, Williams, and Post‡ is widely used by life insurance companies, hospitals, and

From the Biochemical Laboratory of the Metropolitan Life Insurance Company.

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\*Sulfosalicylic acid reagent granules No. 2 are manufactured and sold by Cargille Scientific Inc., New York, N. Y.

†Sugar reagent tablets are manufactured by Ames Company, Inc., Elkhart, Ind.

‡Kingsbury, F. B., Clark, C. P., Williams, G., and Post, A. L.: The Rapid Determination of Albumin in Urine, *J. LAB. & CLIN. MED.* 11: 981, 1925.

clinics. It was necessary in devising the method described herein to show only that the presence of sulfosalicylic acid used in the test for albumin does not interfere with the test for sugar by Benedict's solution. The sugar reagent is sufficiently alkaline to neutralize the acid and it functions as though the sulfosalicylic acid were not present. Two series of urine containing 0.25, 0.5, 0.75, 1.0, and 1.5 per cent glucose were run, using in one case 0.8 ml. of water and in the other 0.8 ml. of a 4.5 per cent solution of sulfosalicylic acid plus 5 ml. of Benedict's solution. The two series were heated simultaneously in a boiling water bath for five minutes. Upon cooling, no difference in the results could be observed.

#### SUMMARY

A rapid method for detecting albumin and sugar in the same measured sample of urine is described.

## BOOKS RECEIVED

---

- Leprosy.** By *Sir Leonard Rogers*, K.C.S.I., C.I.E., M.D., F.R.C.P., F.R.C.S., F.R.S., I.M.S. (Retired); Late Physician and Lecturer, London School of Tropical Medicine; Professor Pathology, Calcutta Medical College; and *Ernest Muir*, C.I.E., M.D., F.R.C.S. (Edin.); Medical Secretary, British Empire Leprosy Relief Association; Late Research Worker in Leprosy at the School of Tropical Medicine and Hygiene, Calcutta. The Williams & Wilkins Company, Baltimore, 1946. Third edition. Price \$7.00. Cloth with 273 pages and 88 illustrations.
- Essentials of Medicine.** By *Charles Phillips Emerson, Jr.*, A.B., M.D.; Assistant Professor of Medicine, Boston University School of Medicine; Associate Member Robert Dawson Evans Memorial and Massachusetts Memorial Hospitals; Research Fellow in Medicine, Harvard Medical School; Resident Physician, Thorndike Memorial Laboratory, Boston City Hospital; *Jane Elizabeth Taylor*, R.N., B.S., M.Ed.; Nursing Education Consultant, U. S. Public Health Service; Formerly Assistant Professor of Medical Nursing, Yale University School of Nursing; and Assistant in Charge of Medical Nursing, New Haven Hospital. J. B. Lippincott Company, Philadelphia, 1946. Price \$3.50. Cloth with 674 pages.
- Practical Malariology.** By *Paul F. Russell*, M.D., M.P.H.; Colonel, M.C., A.U.S.; Parasitology Division, The Army Medical School; Field Staff, International Health Division, Rockefeller Foundation (on leave); *Luther S. West*, Ph.D., Head of Biology Department, Northern Michigan College of Education; Major, Sn.C., A.U.S. (Reserve); formerly Entomologist, Parasitology Division, Army Medical School; and *Reginald D. Maxwell*, Sc.D., Professor of Zoology, Syracuse University, Syracuse, N. Y.; formerly Captain, Sn.C., A.U.S.; Protozoology Section, Parasitology Division, Army Medical School. Prepared under the auspices of the Division of Medical Sciences of the National Research Council. W. B. Saunders Company, Philadelphia, 1946. Price \$8.00. Cloth with 684 pages.

# MORPHOLOGIC CHANGES IN THE LYMPHOCYTES OF PERSONS EXPOSED TO IONIZING RADIATION

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LOS ALAMOS, N. M.

**E**XAMINATION of supravital preparations of the blood of persons exposed to ionizing radiations and toxic chemicals at the Los Alamos Scientific Laboratory has revealed the presence of an unusually large number of refractive neutral red bodies in the cytoplasm of the circulating lymphocytes. These bodies are also present in smaller numbers in lymphocytes of unexposed persons. They can be distinguished from intracellular vacuoles which also stain with neutral red. The increase in neutral red bodies is evident even when exposure to radiation is within the tolerance range, eq., not greater than 0.1 roentgen of x-rays or gamma rays per day delivered to the entire body. The neutral red bodies are found in increased numbers after acute exposure to doses of ionizing radiation which do not affect the total white blood cell count, the total lymphocyte count, or the differential blood cell picture.

## METHODS AND MATERIALS

This study covers a two-year period from 1944 to 1946. All of the subjects have been employed at the Los Alamos Scientific Laboratory. Except for those in control group, all persons have been engaged in work which has resulted in exposure to ionizing radiation or toxic chemicals. With rare exceptions, the exposure was limited to one type of ionizing radiation or to one kind of chemical. The subjects included in this report were carefully selected for uniformity of sex and age. The conditions under which the study was carried out were kept as uniform as possible. The subjects were healthy, adult men, the great majority of whom were between 20 and 30 years of age. All were in residence at the Los Alamos project. All subjects were essentially of the same economic status. Each blood sample was taken in the morning between eight-thirty and ten o'clock. Excluded from this series was any subject showing evidence of illness (except for a group of ambulatory controls specifically chosen for upper respiratory infections), extreme fatigue, or hematologic abnormalities of known or unknown etiology (except radiation).

As far as can be ascertained, dissimilarities between the control and the exposed subjects were limited to occupation and number of blood counts per subject. All exposed persons were engaged in laboratory work of some sort,

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From the Los Alamos Scientific Laboratory.

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while the occupations of the control group ranged from physical work performed out-of-doors to clerical work. Relatively few persons in the control series worked under conditions comparable to those under which the exposed subjects worked. As far as the number of blood counts per subject is concerned, not more than one or, at the most, two blood counts were obtained for each control, while monthly or bimonthly blood counts were done on the exposed personnel. In many cases this was the only blood count which a control subject had experienced. The emotional response in some of these individuals cannot be ruled out as a factor which may have modified their blood pictures.

Every practical means was used to minimize technical errors and to reduce the statistical variation of individual counts. All pipettes and hemocytometer chambers used in this study were standardized by the United States Bureau of Standards. The red blood cell count was determined by examining two sides of a hemocytometer chamber filled with well-mixed diluted blood from a single pipette. Both sides of two chambers containing mixed diluted blood from two pipettes were counted to determine the white blood cell count. The hemoglobin content of the blood was estimated by means of the oxyhemoglobin method of Evelyn.<sup>1</sup> The differential leucocyte picture was obtained by examining 300 cells—200 on a dried film of blood stained with Wright's stain, and 100 in supravital preparations. In most cases, the number of refractive neutral red bodies in fifty lymphocytes was counted and recorded.

Since the morphologic change in the lymphocytes reported in this article can be visualized only in supravital preparations, a detailed account of this technique as used in the Los Alamos Medical Laboratory will be given:

Microscopic slides and cover slips are carefully cleaned with potassium dichromate-sulfuric acid solution. They are then rinsed with distilled water, soaked several days in ethyl alcohol, dried, and stored in a dust-free box. A small drop of blood from a fresh stab incision on the finger or lobe of the ear is picked up with a clean cover slip (No. 0). The glass is inverted and allowed to fall on a microscopic slide on which an alcoholic solution of neutral red and Janus green has dried. When the blood has spread between the two surfaces to form a thin film, the cover slip is rimmed with petroleum jelly. The preparation is kept at 10 to 15° C. until just before examination, when it is allowed to come to room temperature.

In order to obtain adequate uptake of the dyes by the leucocytes, it has been necessary to use much stronger solutions of stain than those recommended in the literature.<sup>2</sup> The dye solution used in this laboratory consists of 4.0 ml. of a saturated alcoholic solution of Janus green and 16.4 ml. of a saturated alcoholic solution of neutral red dissolved in 100 ml. of absolute ethyl alcohol freshly prepared by the sodium phthalate method.<sup>3</sup> The alcoholic solution is allowed to flow onto clean microscopic slides and the excess is wiped off immediately. The slides are dried and stored in light-proof, dust-free containers. Freshly stained slides are prepared every forty-eight hours. Presumably, the intensity of scattered sunlight at this altitude (7,300 feet above sea level) modifies the dyes in an unknown manner which makes necessary the use of higher concentrations of staining solutions. The appearance of the cells and





Fig. 3.—Photomicrograph (magnification  $\times 1200$ ) of a supravital preparation of blood cells of a subject exposed to ionizing radiation. There are fifteen refractive neutral red bodies in the lymphocyte at the center of the picture, of which only a few can be seen at this focus. One refractive body is clearly seen at one o'clock. The apex of the V-shaped shadow at three o'clock is formed by a cluster of five refractive neutral red bodies. The remainder of this shadow is composed of mitochondria and three neutral red bodies, all of which are partly out of focus.



Fig. 4.—Photomicrograph of same field as shown in Fig. 3, through phase optical system (1.8 mm. oil immersion objective with integral diffraction plate, type 1B-0.25). All of the fifteen refractive bodies in the lymphocytes can be identified in this picture because of the great depth of focus of the phase microscope. Note the deep shadows cast by the cluster of refractive bodies at three o'clock and by the single body at one o'clock seen in Fig. 3. The light shadow extending from eight o'clock to eleven-thirty o'clock is produced by mitochondria.



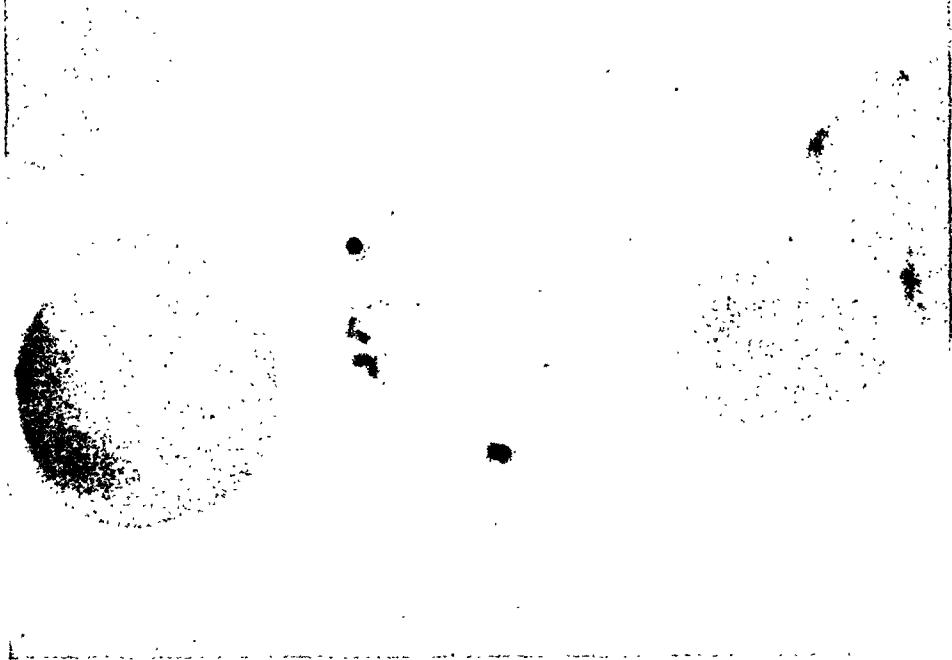


Fig. 1.—Photomicrograph (magnification  $\times 1200$ ) of a supravital preparation of blood cells of a subject recently exposed to small repeated doses of radiation. Refractive neutral red bodies are seen at five-thirty and nine-thirty o'clock in the lymphocyte at the center of the print. The cluster of shadows between the two bodies are mitochondria.

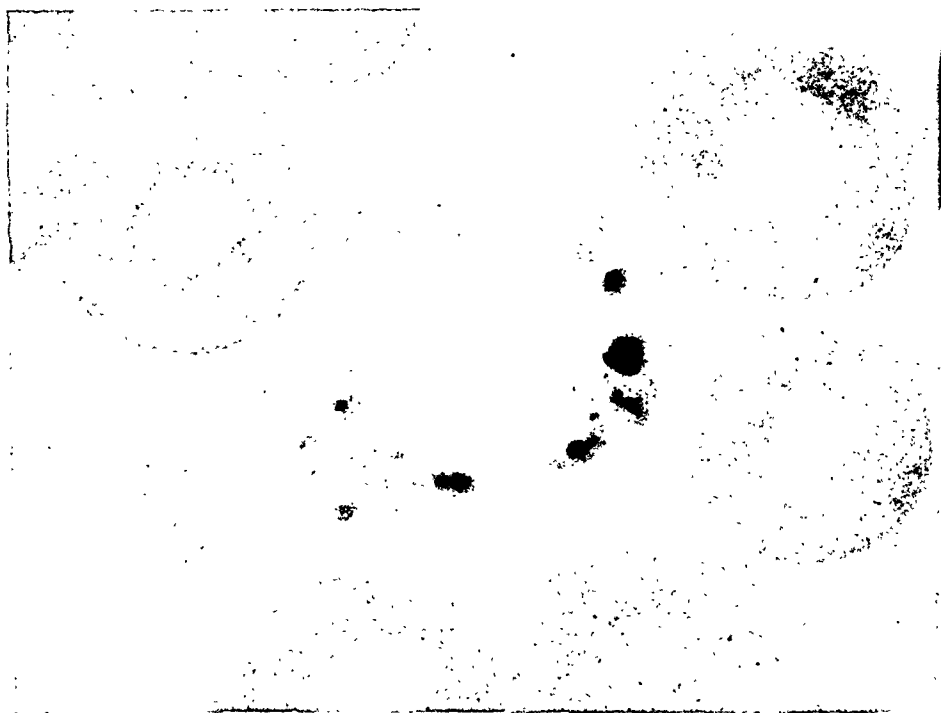


Fig. 2.—Photomicrograph (magnification  $\times 1200$ ) of a supravital preparation of blood from a subject after exposure to small doses of gamma rays for a period of two months. There are fifteen refractive neutral red bodies in the lymphocyte, some of which are almost out of focus. The large shadow at three o'clock is formed by two refractive bodies. The other shadows in the cytoplasm are produced by mitochondria.

uptake of dye by leucocytes resulting from the use of slides prepared with these strong solutions do not differ in any way from that observed in other laboratories where more dilute solutions of supravital dyes are employed.

### RESULTS

It was observed that the lymphocytes of persons exposed to ionizing radiations and toxic chemicals contained more refractive neutral red bodies than did the cells in the blood of unexposed subjects. These bodies, previously described in normal lymphocytes by Gall,<sup>4</sup> were irregularly placed throughout the cytoplasm. They were irregular in shape, varied considerably in size, and had a more decided brick-red color than did the neutral red-staining vacuoles (Figs. 1 to 3). There was no increase in number or size on standing twelve hours at 10 to 15° C. The number of refractive bodies per cell varied from zero to more than thirty. The refractiveness of these bodies resembles that of the granules in cells of the granulocytic series and distinguishes them from vacuoles and nonrefractive bodies which also stain with neutral red dye.

Examination of the same blood samples stained with Wright's, Giemsa's, and peroxidase stains revealed no morphologic abnormalities of the lymphocytes similar to that described.

By using contrast microscopy, additional information was gained about the structure of the refractive neutral red bodies. If individual cells in a supravital preparation were examined first through an ordinary microscope and then through a phase microscope, it was observed that the refractive neutral red bodies showed a high phase contrast while the nonrefractive and vacuolar neutral red-staining bodies showed a low phase contrast (Fig. 4). This indicates that the density of the neutral red bodies is high and that they probably exist in the solid or granular phase. The mitochondria are the only other structures in the lymphocytes that show a high phase contrast. These bodies are not easily confused with the neutral red bodies under the contrast microscope since they are smaller and their green color is poorly transmitted through the phase optical system, while the red color of the refractive bodies is well transmitted.

The increase of the neutral red bodies in the lymphocytes of the exposed groups represents essentially a quantitative rather than a qualitative change. The modification in the numbers and distribution of neutral red bodies in lymphocytes for subjects variously exposed to radiation is shown in Fig. 5. In order to facilitate the demonstration of lymphocytic changes of this nature, it was decided to differentiate between cells which contained an abnormally large number of refractive neutral red bodies and those which did not. It was agreed, therefore, on a completely arbitrary basis, to designate any lymphocyte with five or less neutral red bodies as "normal" and to consider any cell with six or more bodies as "abnormal." This terminology will be used in the remainder of this article.

One thousand sixty-four hematologic studies on 364 subjects have been analyzed. Fig. 7 is a scatter diagram showing the percentage of abnormal lymphocytes in all subjects divided into control and exposure groups. The explanation of the type and degree of exposure of each group is given in Table I.

TABLE I. TYPE AND DEGREE OF EXPOSURE OF GROUPS MENTIONED IN SCATTER DIAGRAMS

GROUP	NUMBER OF PERSONS IN GROUP	DESCRIPTION OF WORK	DEGREE OF EXPOSURE
Group A			
Unexposed			
Class I			
Military personnel	62	Physical work out-of-doors	None
Civilian personnel	40	Indoor work ranging from clerical and administrative jobs to theoretical scientific work	None
Ambulatory clinic patients with upper respiratory infection	8	Varied	None
Class II			
Laboratory technicians and scientists	10	Laboratory work	None, but incidental exposure to radiation cannot be excluded completely
Skilled craftsmen	49	Plumbing, carpentry, electrical work, and machine shop work	No known exposure to radiation; plumbers exposed to lead oxide fumes within tolerance limits; machinists exposed to metal fumes
Group B			
External radiation			
Class I			
	25	Laboratory work	Incidental exposure to external radiation; average exposure less than 0.3 to 0.5 r per month
Class II			
	41	Laboratory work	Intermittent exposure chiefly to natural sources; average exposure 1 r per month or less
Class III			
	25	Laboratory work	Consistent exposure chiefly to radiation from accelerating equipment (cyclotron, Van de Graaff, etc.); average exposure 1.5 r per month or less; rarely did dose in any given day approach 0.1 r
Class IV			
	22	Laboratory work	Consistent exposure over six-month period to strong radioactive sources; monthly exposure 3.0 r or less, with certain persons exceeding this value for a single month; daily exposures frequently exceeded 0.1 r and occasionally reached 0.5 r
Group C			
Radioactive material A			
Class I			
	8	Laboratory work	Contact with radioactive material A, an alpha particle emitter; no deposition of material within body according to reasonably sensitive tests

TABLE I—CONT'D

GROUP	NUMBER OF PERSONS IN GROUP	DESCRIPTION OF WORK	DEGREE OF EXPOSURE
Class II	17	Laboratory work	Contact with radioactive material A; some deposition of material in the body, but average amount below maximum permissible value
Radioactive material B Class I	29	Laboratory work	Contact with radioactive material B, an alpha particle emitter; no deposition of material in the body according to moderately sensitive test
Class II	6	Laboratory work	Contact with radioactive material B; deposition of material in the body approaching maximum permissible amounts
Group D Uranium	20	Laboratory and plant work	Mild to moderate exposure to uranium chiefly as oxide fumes

This exposure refers to the period 1943 to 1946 and does not take into consideration the previous exposure record of the subject. In practically all cases except those in Radiation Groups II and III, previous exposure to toxic chemicals or radiation was negligible. Each point in the scatter diagram represents the percentage of abnormal lymphocytes for a single subject. In the case of most of the controls, this point is based on one examination; in the case of the exposed subjects, the point represents the average of many (in one instance, thirty) examinations. Where radiation exposure was intermittent it was impossible to correlate the time of the hematologic examinations with the time of the exposure. For purposes of comparison, Figs. 6, 8, 9, and 10 show scatter diagrams of the total white blood count, the total abnormal lymphocyte count, the total lymphocyte count, and the percentage of lymphocytes in the differential white blood cell count for each exposure group. As in the case of Fig. 7, each point represents an average figure for all examinations on a single individual.

Figs. 11 and 12 show the increase in percentage of abnormal lymphocytes in persons accidentally exposed to single, large, instantaneous bursts of general body radiation. It is unfortunate that previous determinations of the percentage of abnormal lymphocytes were not made on these subjects. A complete hematologic report of these cases will be presented in a forthcoming article. Single exposures to general body gamma radiation which did not exceed 5 roentgens and which were delivered over a period of several hours produced no increase in the percentage of abnormal lymphocytes.

Similar increases in abnormal lymphocytes have been observed in rabbits and cows exposed to large doses of ionizing radiation. Morphologic changes of this type in the lymphocytes of mice and rats exposed to radiation could

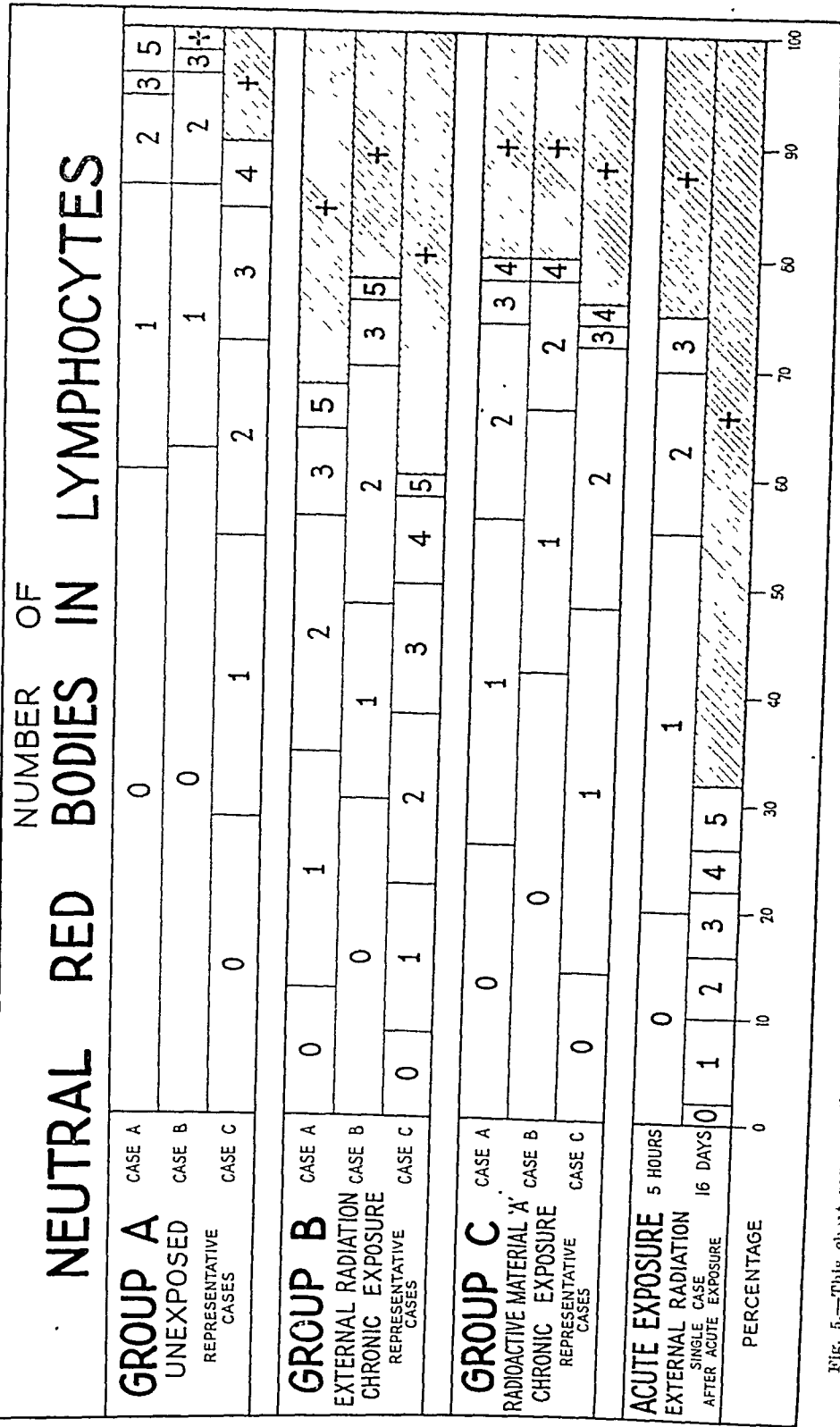


Fig. 5.—This chart arranges the lymphocytes of representative individuals in various exposure groups according to the number of refractive neutral red bodies which they contain. The bands extending across the page represent 100 per cent of the lymphocytes for an individual. The scale of lymphocytes in percentage is shown at the bottom of the chart. The numbers in each segment of a band denote the number of refractive bodies in that fraction of the lymphocytes containing more than five bodies. The lymphocytes containing more than five bodies were lumped together in one group designated by a plus sign. Thus, in Case A, Group A, 10 per cent of the lymphocytes contained no neutral red bodies; 25 per cent, one body; 8 per cent, two bodies; 2 per cent, three bodies; and 5 per cent, five bodies.



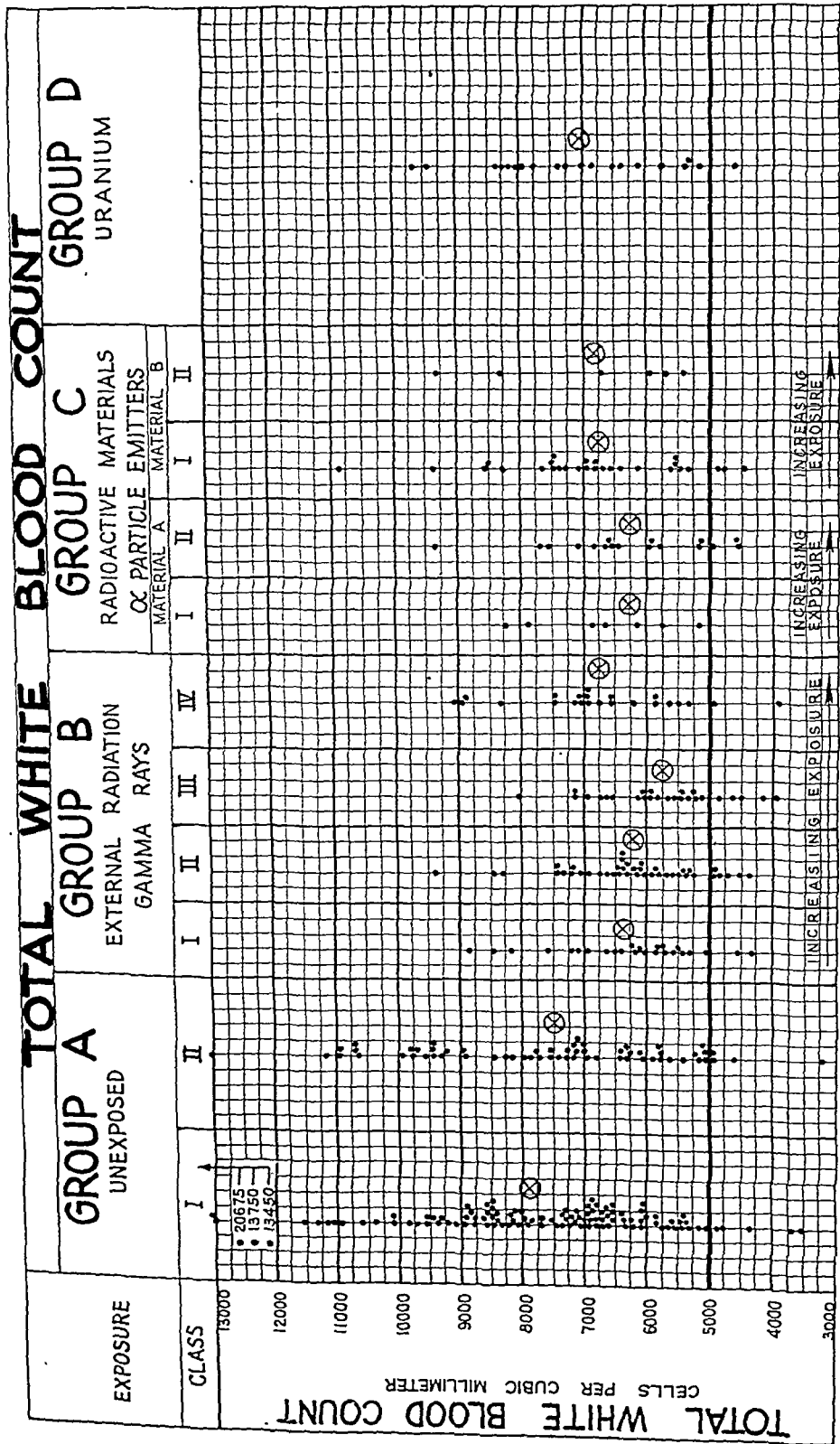


Fig. 6.—Scatter diagram showing total leucocyte count for individuals in each exposure group. The cross in each column represents the average leucocyte count for this group.

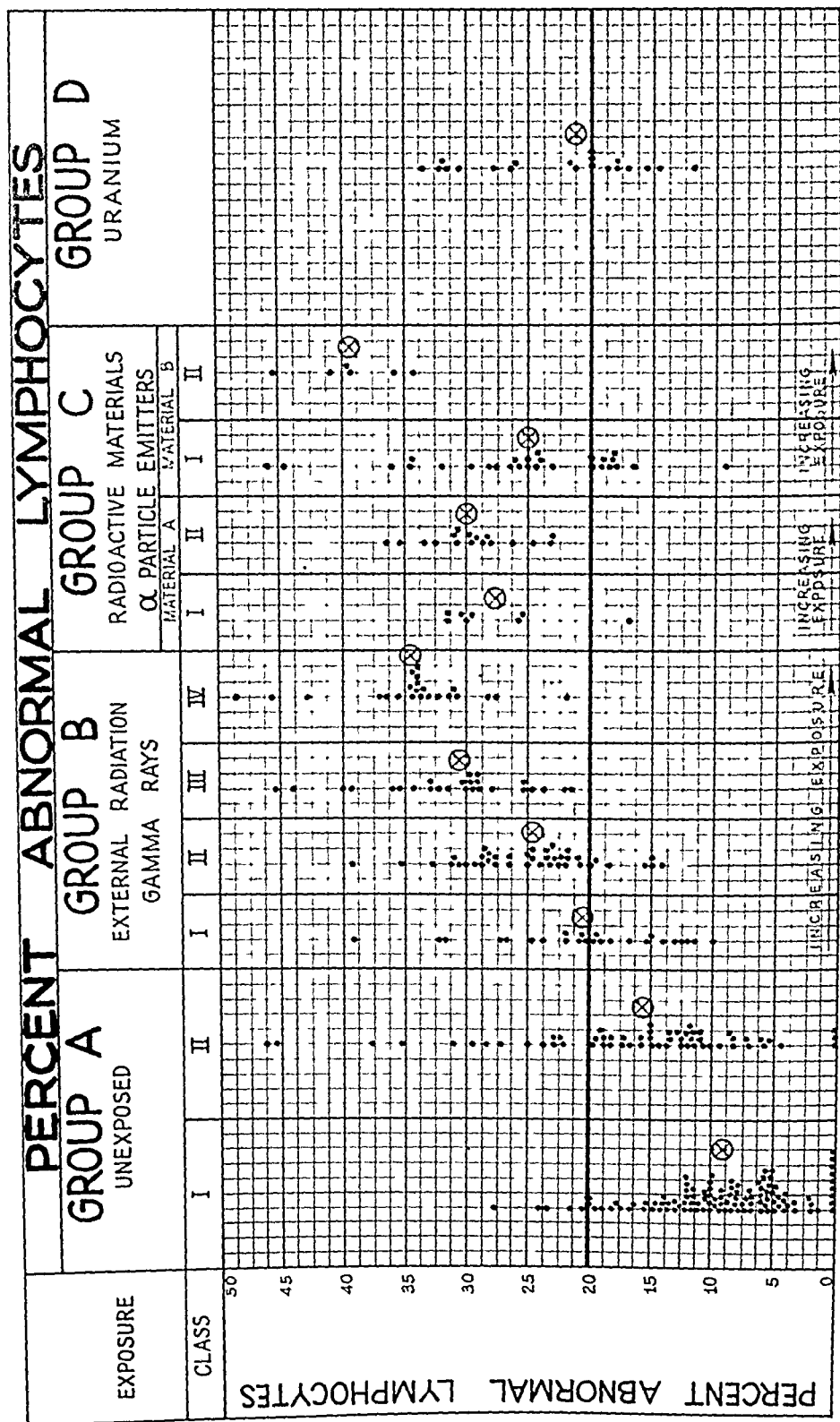


Fig. 7.—Scatter diagram showing the percentage of abnormal lymphocytes for persons in each group. The cross in each column represents the average percentage of abnormal cells for each group.

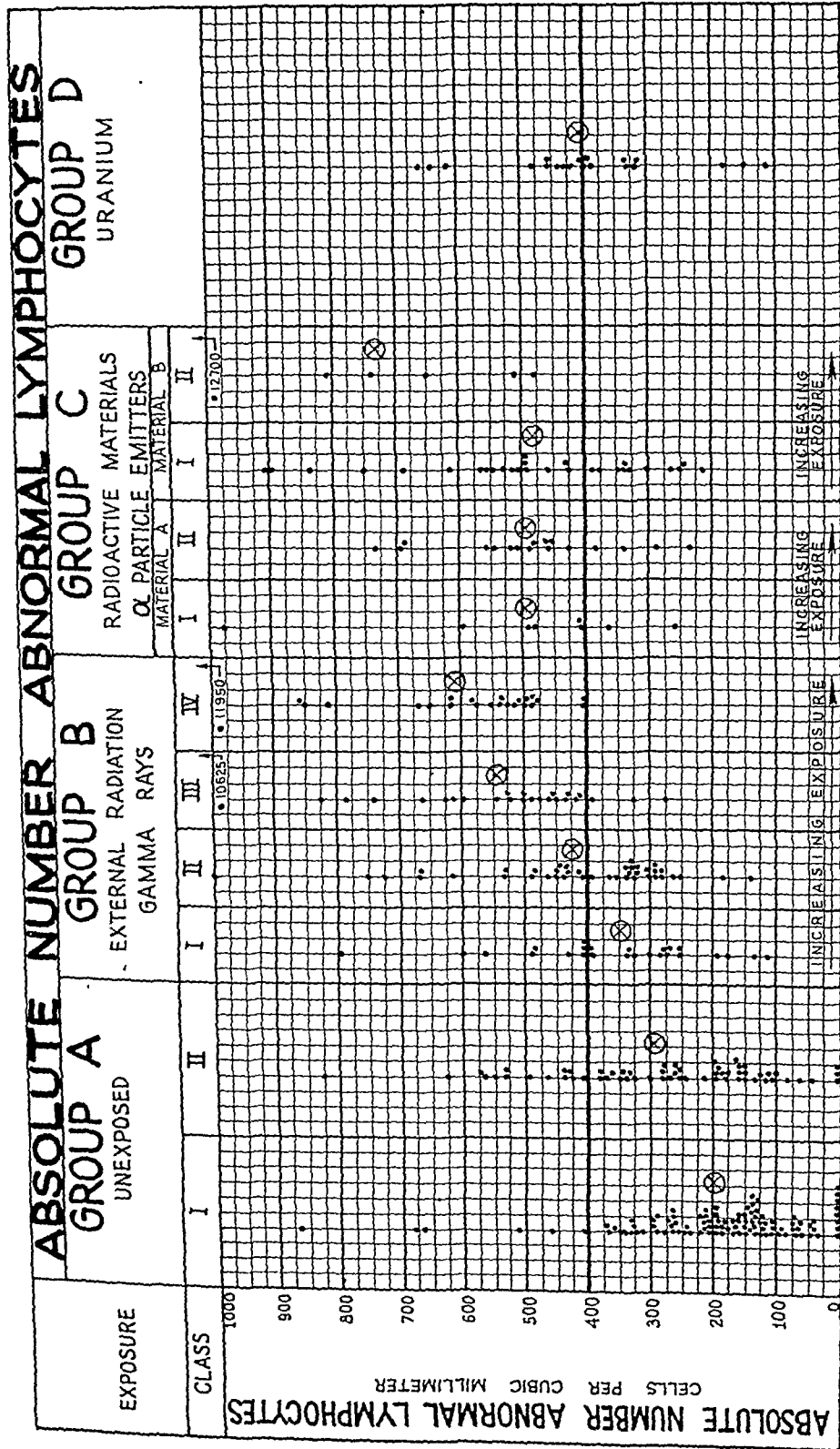


Fig. 8.—Scatter diagram showing the absolute number of abnormal lymphocytes for persons in each group. The cross indicates the average abnormal cells per cubic millimeter for each group.

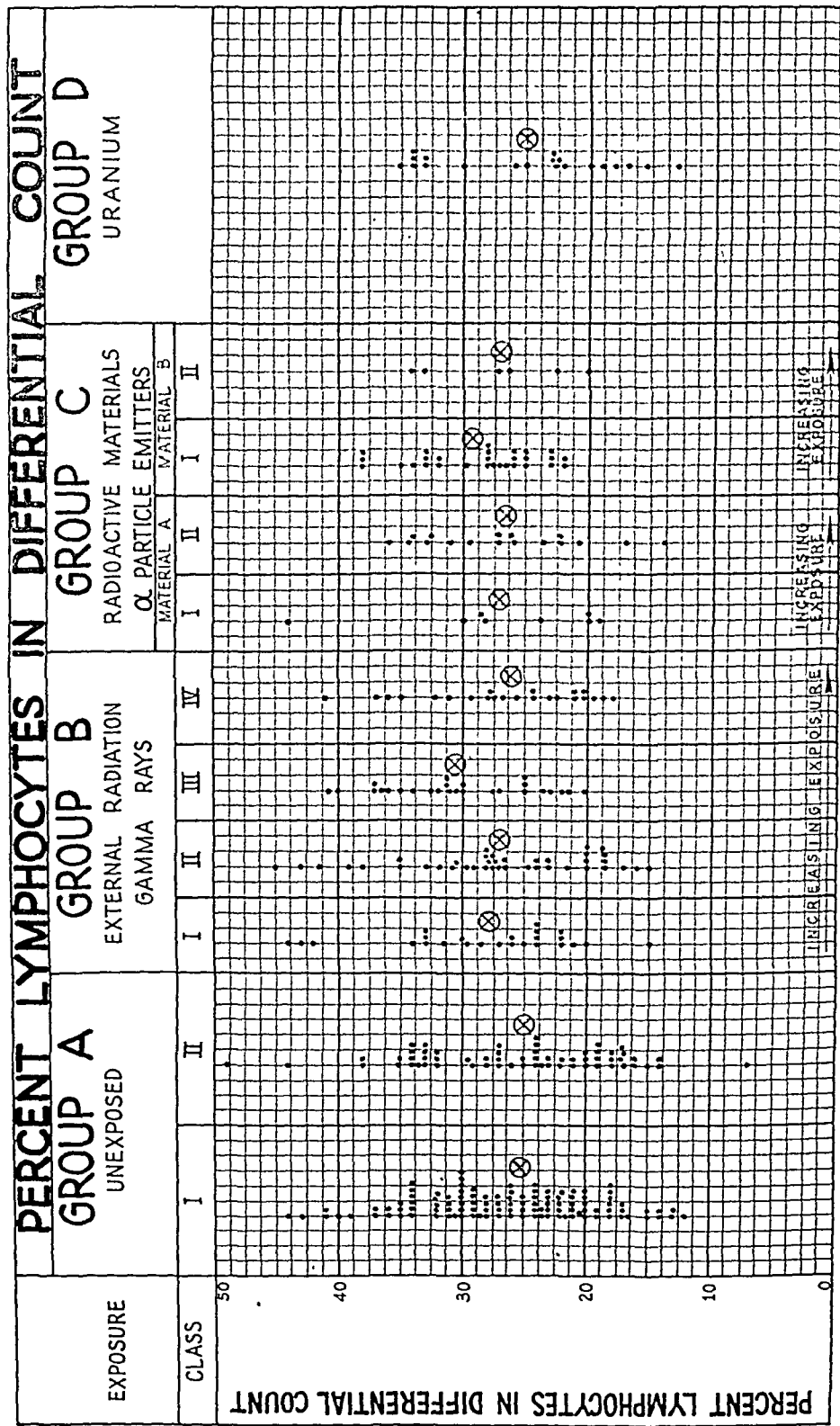


Fig. 9.—Scatter diagram showing the percentage of lymphocytes in the differential count for persons in each group. The cross indicates the average for each group.

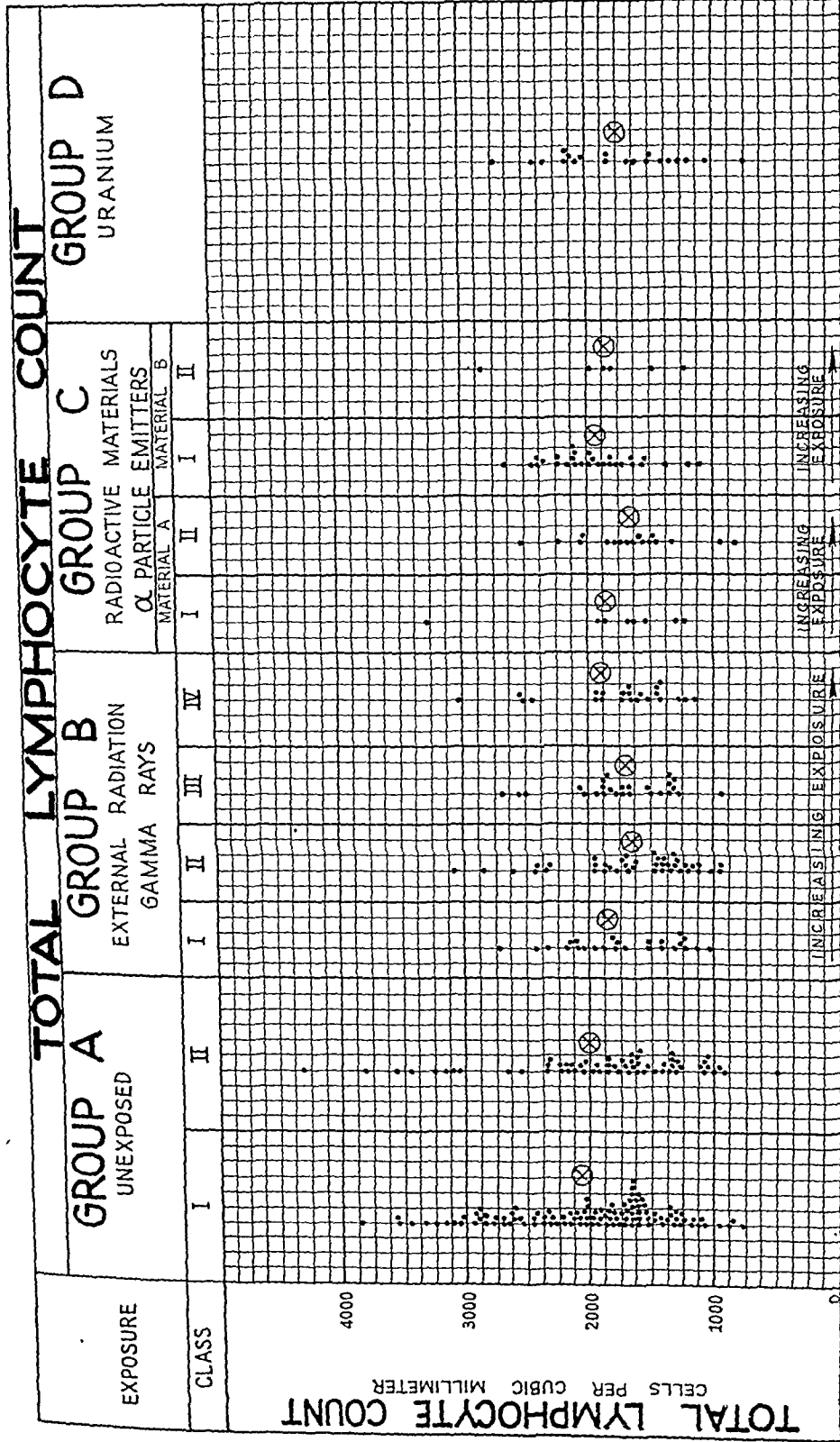


Fig. 10.—Scatter diagram showing the total lymphocyte count for subjects divided into groups. The cross denotes the average for the group.

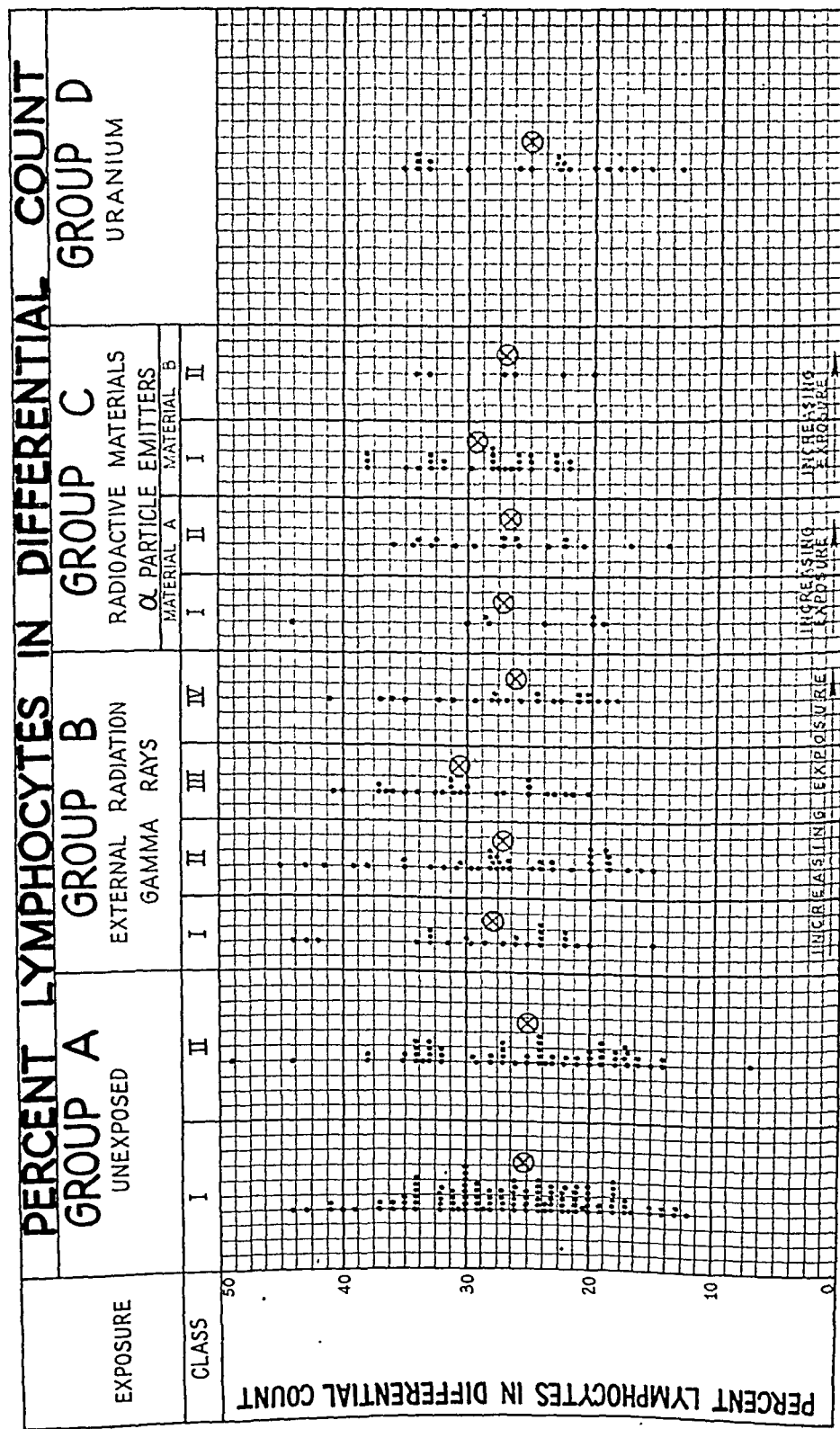


Fig. 9.—Scatter diagram showing the percentage of lymphocytes in the differential count for persons in each group. The cross indicates the average for each group.

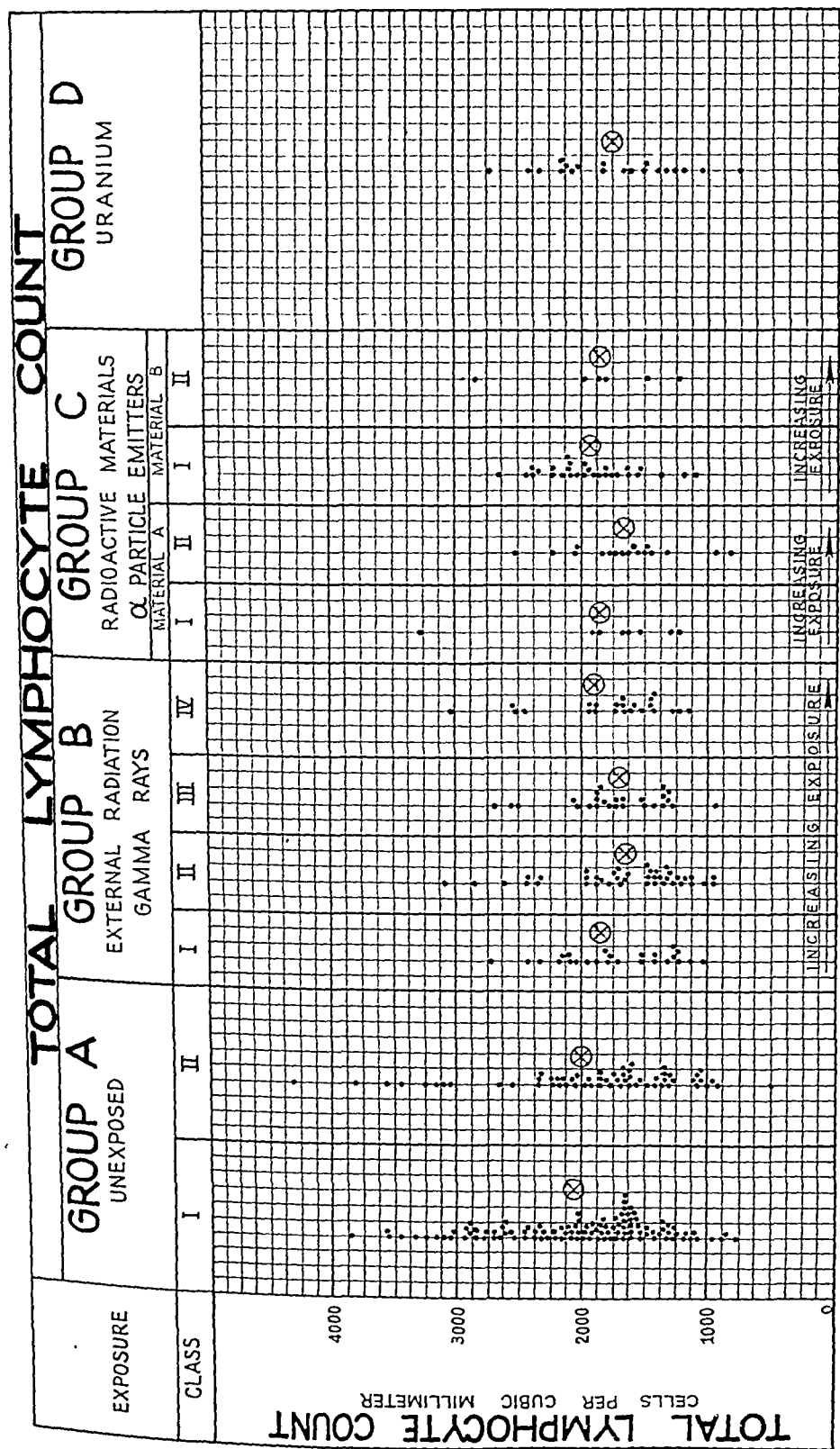


Fig. 10.—Scatter diagram showing the total lymphocyte count for subjects divided into groups. The cross denotes the average for the group.

# INCREASE IN REFRACTIVE NEUTRAL RED BODIES IN LYMPHOCYTES AFTER ACUTE EXPOSURE

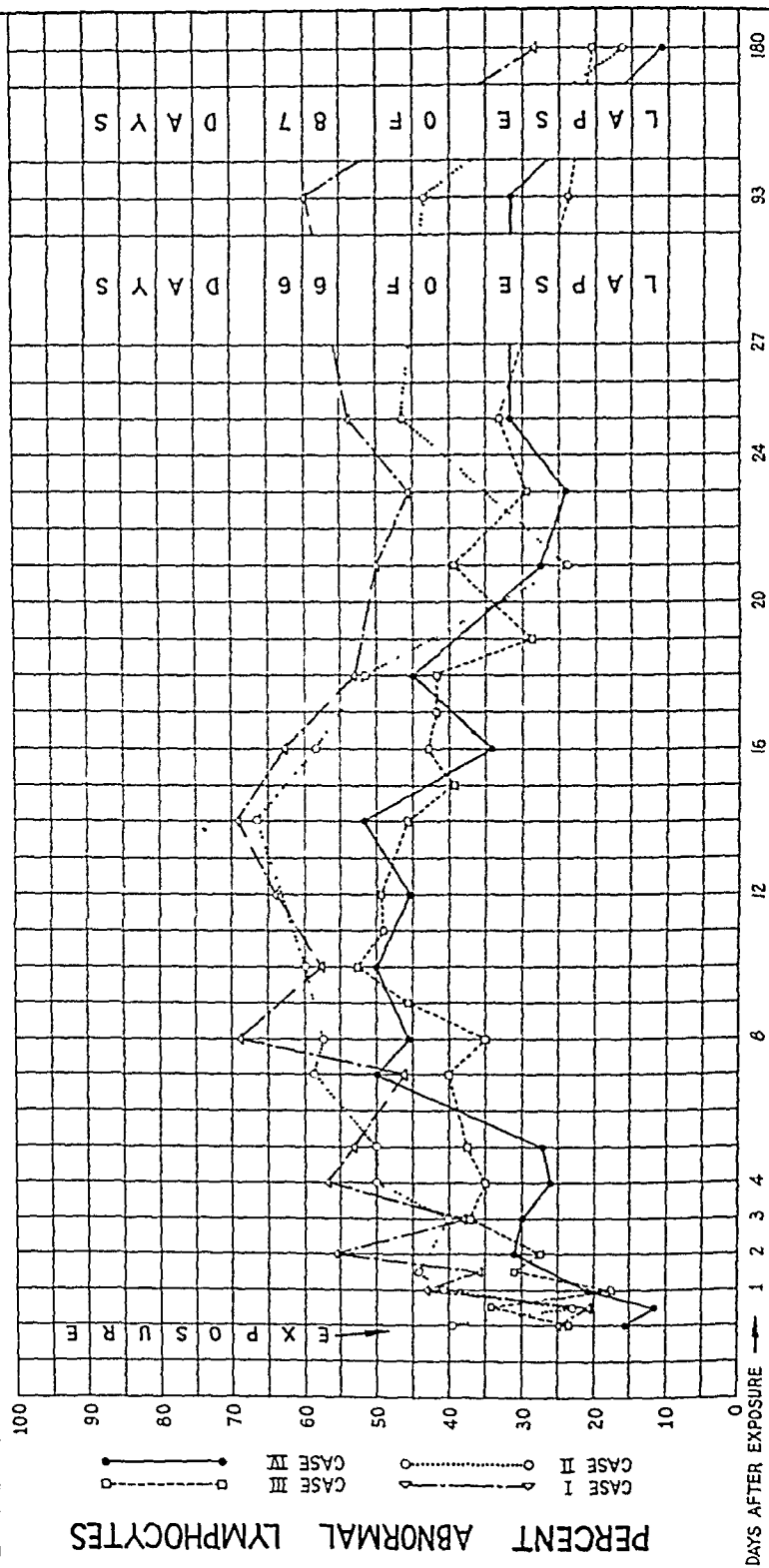


Fig. 11.—Chart showing the percentage of abnormal lymphocytes. In four subjects exposed to an instantaneous burst of total body radiation. All subjects showed high initial total leucocyte counts, but subsequent changes in the blood counts indicated evidence of mild transient bone marrow injury only in Cases I and II.



# INCREASE IN REFRACTIVE NEUTRAL RED BODIES IN LYMPHOCYTES AFTER ACUTE EXPOSURE

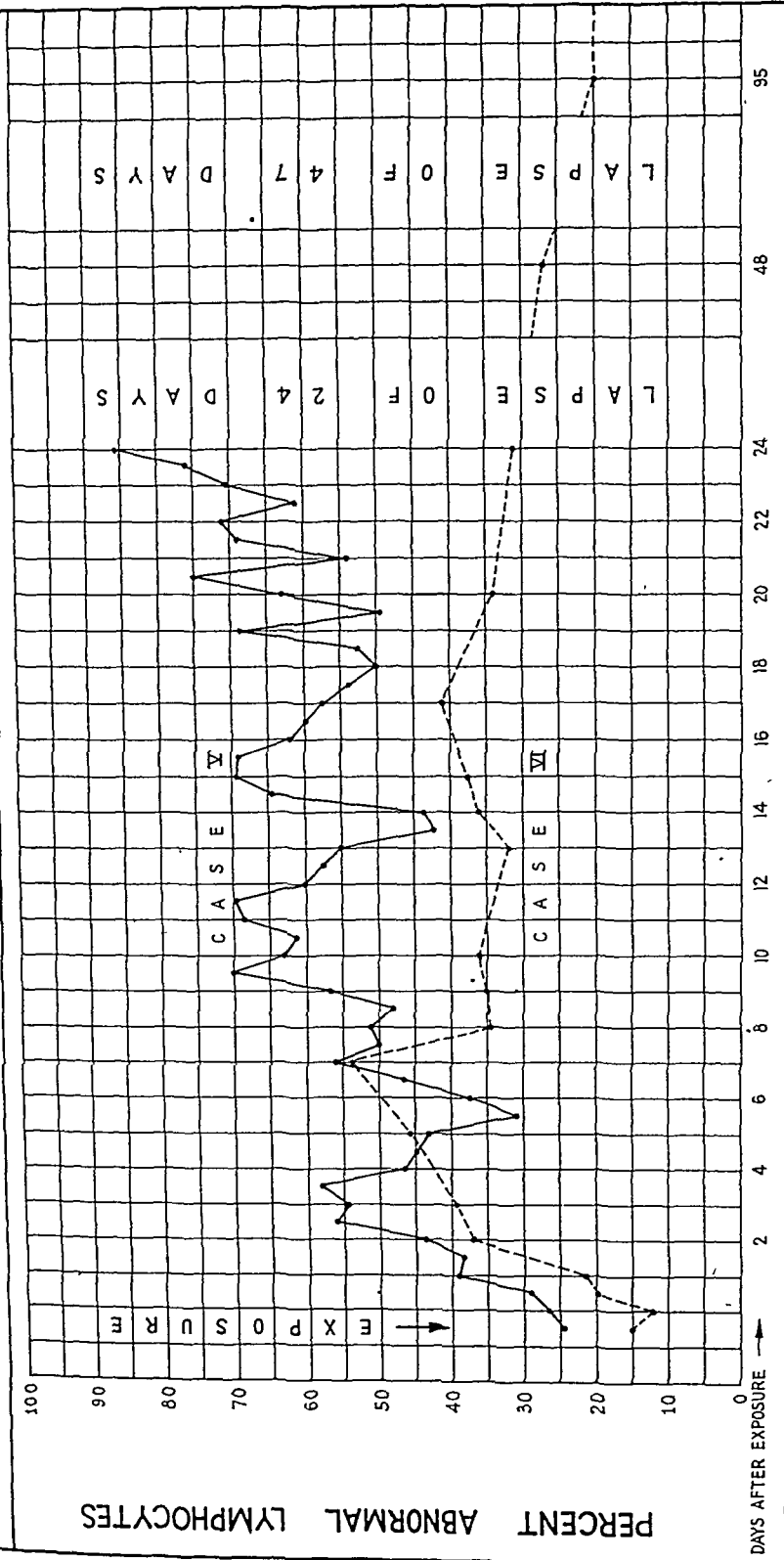


Fig. 12.—Chart showing the percentage of abnormal lymphocytes in two subjects receiving brief exposure to large doses of ionizing radiation. Case V received a lethal amount of radiation. Case VI was exposed to a relatively small dose of radiation which produced no other changes in the blood picture.

not be detected because the large amounts of neutral red dye normally taken up by the cytoplasm of the lymphocytes interferes with the identification of the refractive bodies.

#### DISCUSSION

A survey of the literature has failed to disclose previous descriptions of similar morphologic changes in lymphocytes after *in vivo* exposure to small repeated doses of ionizing radiation. Morphologic changes in living cells of various kinds have been reported following exposure to single large doses of radiation. Prigosen<sup>5</sup> has reported the appearance of neutral red bodies in irradiated tumor cells. Recently Schrek<sup>6</sup> described an increase in cytoplasmic vacuoles in dark-field preparations of lymphocytes after *in vitro* and *in vivo* exposure to x-rays.

Inspection of the scatter diagrams in this report shows that there is an increase in abnormal lymphocytes in the persons exposed to ionizing radiation. The percentage and total number of abnormal cells can be correlated with the magnitude of chronic exposure. The significance of the data presented in these diagrams is evident without further statistical treatment. There are several features of these diagrams, however, which require further discussion.

A significant difference between the controls (Group A, Class I) and the groups exposed to radiation (Group B, Classes I, II, III, and IV) was observed only for total leucocyte count, the proportion and absolute numbers of abnormal lymphocytes. In the case of the total white blood cell count, the difference can be demonstrated by comparing the average counts for each group. Since most of the total counts of all groups lie between 5,000 and 9,000 cells per cubic millimeter, however, the value of any individual count has little significance in determining the exposure group into which the subject falls. On the other hand, the difference in the percentage of abnormal cells and absolute abnormal cell counts between control and exposed subjects is reflected in the value for an individual subject. Most of the controls have less than 20 per cent abnormal cells or 400 abnormal cells per cubic millimeter, while all of the more consistently exposed subjects show percentages and total numbers of abnormal cells above these levels. There is so little overlap of points for exposed and control groups that the appearance of few or many abnormal cells has more than chance significance in determining the exposure of any given individual.

A difference in percentage and absolute numbers of abnormal lymphocytes also exists between the controls and the groups exposed to certain chemicals. In this respect, it should be emphasized that the biologic action of natural uranium is due to its chemical rather than its radioactive properties. Thus the increase in percentage of abnormal lymphocytes in Group D is probably related to the chemical effect of uranium. The increase in percentage of abnormal cells in Group A, II (Fig. 7) is added evidence pointing to the fact that exposure to toxic chemicals changes the percentage and the absolute number of abnormal cells, since nine of the fourteen points above the 20 per cent abnormal cell level represent plumbers who were frequently exposed to lead fumes. These subjects have shown no clinical or laboratory evidence of plumb-

ism except for an occasional mild degree of basophilic stippling of the red blood cells. An increase in abnormal lymphocytes was also found in persons working with industrial nonradioactive chemicals besides uranium and lead.

It is evident from Figs. 11 and 12 that exposure to a single large dose of ionizing radiation increases the proportion of abnormal cells in the circulating blood. The response of the lymphocytes of the four subjects in Fig. 11 is essentially the same, although the radiation dosage of the subjects differed by a factor of ten. It must be concluded, therefore, that the increase in abnormal lymphocytes is not proportional to the dosage when administered as a single brief exposure.

#### SUMMARY

1. Analysis of the total leucocyte counts of persons chronically exposed to ionizing radiation and toxic chemicals shows a significant statistical decrease in the exposed groups.

2. Analysis of the absolute number and of the percentage of lymphocytes in the differential counts of the same persons shows no significant change.

3. Morphologic study of supravital preparations of blood cells of persons chronically and acutely exposed to ionizing radiation indicates a striking increase in the number of refractive neutral red bodies in the cytoplasm of the circulating lymphocytes. An increase in neutral red bodies is also found in persons working with toxic chemicals.

4. These neutral red bodies have high density and may be considered to be granules. They have not been identified in fixed preparations.

#### REFERENCES

1. (a) Evelyn, K. A.: Stabilized Photoelectric Colorimeter With Light Filters, *J. Biol. Chem.* 115: 63, 1936. (b) Evelyn, K. A., and Cipriani, A. J.: Photoelectric Microcolorimeter, *J. Biol. Chem.* 117: 365, 1937. (c) Evelyn, K. A., and Malloy, H. T.: Microdetermination of Oxyhemoglobin, Methemoglobin, and Sulfhemoglobin in Single Sample of Blood, *J. Biol. Chem.* 126: 655, 1938.
2. Fieser, Louis F.: *Experiments in Organic Chemistry*, ed. 2, Boston, 1941, D. C. Heath & Co.
3. Cunningham, R. S., and Tompkins, E. H.: *The Supravital Method of Studying Blood Cells*, Downey's Handbook of Hematology, vol. I, New York, 1938, Paul B. Hoeber, Inc.
4. Gall, E. A.: A Previously Undescribed Granule Within the Lymphocyte, *Am. J. M. Sc.* 191: 380, 1936.
5. Prigosen, R. E.: Vital Staining of Tumor Cells After Roentgen Rays, *J. Cancer Research* 8: 305, 1924.
6. Schrek, Robert: Dark-field Observations on Lymphocytes Exposed to X-rays and Other Injurious Agents, *Proc. Soc. Exper. Biol. & Med.* 64: 381, 1947.

# STUDIES ON LYMPHOGRANULOMA VENEREUM: EVALUATION OF THE COMPLEMENT FIXATION TEST WITH LYGRANUM

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IN THE development of our knowledge of most infectious diseases, progress has been expedited by improvements in the methods available for diagnosis, particularly in the case of latent infections or in the frank disease when symptomatology and clinical signs are not distinctive. Thus in lymphogranuloma venereum Frei's discovery<sup>1</sup> of the intradermal sensitivity of infected individuals to lymphogranulomatous bubo pus constituted a great advance since it enabled a diagnosis to be made on grounds other than the clinicopathologic basis set forth by Durand, Nicolas, and Favre.<sup>2</sup> By means of this cutaneous reaction a variety of lesions other than those of the genitals and regional lymph nodes were linked to a common background of lymphogranulomatous infection; in some instances it has been possible later to confirm the diagnosis by the actual isolation of the etiologic agent from pathologic material.<sup>3</sup> Until recently, however, a serious deterrent to the routine use of the Frei test in large-scale investigations (which are highly desirable for a more complete understanding of the epidemiology of the disease) was the fact that to the great majority of clinicians there was not readily available human bubo pus of satisfactory specific activity and free from contamination with antigens derived from other infectious organisms.

Several preparations of tissues experimentally infected with virus, such as brain tissue from different animal species,<sup>4</sup> have been suggested and employed as a substitute for human bubo pus. These, while containing sufficient specific antigen to elicit a positive Frei reaction, also include relatively large amounts of foreign tissue which may evoke a nonspecific inflammatory response. This makes interpretation of the test difficult unless it is compared with the result of simultaneous inoculation of normal tissue emulsion as control. Furthermore, allergic hypersensitivity to the accompanying brain tissue, whether inherent in the individual or acquired only after repeated skin testing, may sometimes obscure any specific reaction to the virus antigen.<sup>5</sup> It should be pointed out, however, that while no control material is inoculated in tests with human bubo pus and any reaction following its introduction is taken as evidence of a positive response, there is little reason to believe that the skin of occasional individuals may not be irritated by injection of certain components of such pus irrespective of the presence of, and reaction to, lymphogranuloma antigen.

A new and improved reagent (Lygranum) for the Frei test<sup>6</sup> has been developed as a result of the observation that the etiologic agent of lymphogranuloma venereum will proliferate luxuriantly when introduced into the

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yolk sac of the embryonated hen's egg.<sup>7</sup> The enormous numbers of elementary bodies and other developmental forms of the virus<sup>8</sup> thus obtained have been readily separated from the bulk of the host tissue by differential centrifugation, and the infective properties suitably destroyed while preserving the antigenic activity. In the experience of several groups of investigators<sup>9</sup> the results have been eminently satisfactory, with this purified suspension of virus showing diverse features of superiority over mouse brain antigen and being at least equal in the number of positive reactors detected when compared with highly potent samples of human pus. The concentration of antigen in Lygranum preparations can be controlled, so there is no prevailing reason why it should not be available in a form even more potent than the most active specimens of bubo pus. There has been some criticism of Lygranum because of difficulty in interpreting so-called "nonspecific" reactions,<sup>10</sup> consisting largely of erythema without papule formation or, less frequently, small papules elicited in nonlymphogranulomatous persons. It should be emphasized again, that the zone of erythema seen at the site of inoculation of Lygranum S. T. is of no diagnostic significance whatever. Only the presence of a *definite papule of at least 6 mm.* in diameter should be taken as indicating a positive reaction with Lygranum, and *every test should be controlled* with normal yolk sac material injected simultaneously with the specific antigen. The control injection usually provokes a negligible or trivial reaction, but individuals who are sensitive to chick embryo materials may give papules with the control from normal yolk sac as large as those with Lygranum; in such persons the reaction to Lygranum S. T. should not be considered as positive.

The utility of Lygranum is not limited to the intradermal test for it has also been found potent in the detection of complement-fixing antibodies in the serum.<sup>11</sup> While the evidence thus far acquired has shown Lygranum to be a very sensitive indicator when used in this manner as an aid in the diagnosis of infection with lymphogranuloma virus, other studies have demonstrated that cross reactions are obtained with this antigen in complement fixation tests on the sera from patients with trachoma or inclusion blennorrhoea<sup>12</sup> and in certain cases of atypical pneumonia or pneumonitis due to psittacosis or closely related viruses.<sup>13, 14</sup> This is not unexpected since, in spite of obvious differences in modes of transmission and tissue tropisms, the agents responsible for these diseases appear to share many properties, including the possession of a common antigenic factor.<sup>14, 15</sup> It is one of the purposes of this paper to explore the limits of the specificity of Lygranum in the complement fixation test in the light of a comprehensive summary of our experience with several hundred sera derived from persons with a variety of clinical conditions.

#### REAGENTS AND TECHNIQUE

*Antigens.*—Egg-adapted lymphogranuloma virus was employed as inoculum for six- or seven-day chick embryos via the yolk sacs; the latter were harvested shortly prior to, or immediately following, death of the embryo from specific infection, all embryos being candled twice a day. Those yolk sacs, found on examination to contain abundant virus in the absence of other agents cultivable on artificial media, were pooled and ground or shaken with

ten volumes of physiologic saline. For the tests to be reported here, the virus was freed from most tissue material by differential centrifugation and finally suspended in saline containing 0.1 per cent formalin.<sup>11</sup> Control material was prepared from normal yolk sacs in exactly the same fashion. Nigg has found that a useful antigen may be obtained by the simple expedient of treating the crude saline suspension of virus-infected yolk sac with urea or ether.<sup>16</sup> Recently a combination of boiling and treatment with phenol has proved even more satisfactory<sup>17</sup>; not only may the specific complement-fixing activity of the antigen be enhanced thereby, but also yolk sac preparations treated in this way do not show the non-specific fixation with sera from cases of early syphilis (see below). The mechanisms involved are not understood.

In some of the early tests we employed in place of yolk sac virus the supernate obtained by centrifugalizing at low speed a saline suspension of lymphogranulomatous mouse lungs,<sup>18</sup> together with control material from similarly treated normal mouse lung. The results with the two viral antigens were entirely in agreement; we preferred later to use yolk sac antigen since it was more readily available in large amounts, was usually somewhat more potent, and could readily be obtained free from contamination with other organisms. The lungs of mice may contain varying numbers of adventitious bacteria or other agents which might lead to erroneous results, although we have experienced no difficulty from this cause. Finally the handling of intranasally infected lymphogranulomatous mice constitutes a definite hazard in the laboratory.<sup>19</sup> The soluble antigen found in Seitz filtrates of virus-infected yolk sac or mouse lung tissue<sup>20</sup> has also been utilized, but this may be less potent than suspension-containing particulate virus.

For use in complement fixation tests stock antigen from any of the sources mentioned was adjusted by dilution so that it was not anticomplementary, gave definite reactions with known weakly positive lymphogranulomatous sera, and gave no reaction with sera from normal individuals.

*Sera.*—Just before use the sera were inactivated for twenty minutes at 56 or 60° C.; the higher temperature was helpful in reducing the anticomplementary behavior observed on prolonged storage of serum.<sup>21</sup> Serial doubling dilutions were made in physiologic saline. In most cases the lowest initial dilution of serum tested was 1:2, although on a few occasions the undiluted serum was also used. Since during fixation the serum is acting in a final dilution three times that originally introduced, we have recorded the titers as the highest final dilution giving definite specific fixation.

*Complement.*—Guinea pig serum was used, either fresh or stored at -32° C. until needed; it was titrated and diluted just before use. Two units in 0.2 ml. were usually obtained with a 1:20 to 1:30 dilution.

*Performance of Test.*—The following procedure was found to yield satisfactory results while retaining features of simplicity and convenience, permitting the test to be read within two hours after it was prepared. To a series of tubes was added 0.2 ml. of each of the reagents, in the order stated: diluted patient's serum, 2 units of hemolytic complement, specific antigen. A control series was set up in each instance with normal tissue material in place of the specific antigen. In every test controls were also included to exclude anticomplementary or hemolytic action of the antigen or patient's serum, as well as to demonstrate the activity of the complement. After incubation for seventy-five minutes in a 37° C. water bath, to each tube was added 0.2 ml. of the indicator. This volume contained a 3 per cent suspension of sheep corpuscles in saline and 2 units of amboceptor, the cells having been sensitized beforehand by incubation for thirty minutes at 37° C. Readings for fixation (inhibition of hemolysis) were made after thirty minutes longer at 37° C. Only when all controls were satisfactory was a test considered of possible diagnostic significance. The sensitivity of the test can be increased somewhat by carrying out fixation in the refrigerator overnight or by a short period of incubation in the cold following that at 37° C. Prolonged fixation in the cold increases the tendency to nonspecific fixation with normal yolk sac control material, however, so this refinement was used only rarely.

Nearly all sera were tested in serial dilutions at least twice, and the majority were tested several times to check the reproducibility of results. One additional point should be

mentioned here, namely, the desirability of testing serum specimens with reasonable promptness after their collection. If fluid sera are stored without preservative in the refrigerator for many months they frequently suffer deleterious bacterial or fungous contamination; furthermore, the specific titer tends to diminish with aging while the anticomplementary properties increase, making it difficult or impossible to test satisfactorily low dilutions of the serum. This should be borne in mind, since some sera are of low titer even when tested shortly after their withdrawal from the donor.

# PRESENTATION AND DISCUSSION OF EXPERIMENTAL DATA

1. *Tests on Sera from Persons With History or Signs of Overt Lymphogranulomatous Infection and Positive Frei Test.*—We have tested serum specimens from 149 patients falling in this classification, with the results summarized in Table I.

TABLE I. COMPLEMENT FIXATION TESTS ON SERA FROM PERSONS WITH EVIDENCE OF LYMPHOGRANULOMATOUS INFECTION

TYPES OF LESIONS OBSERVED IN PATIENTS	NUMBER TESTED	LYGRANUM COMPLEMENT FIXATION	
		POS.	NEG.
Genital and/or inguinal lesions*	33	33	0
Posterior bowel lesions†	61	60	1
Other lesions‡	55	54	1
Total	149	147	2

\*This group includes early or chronic lesions of genitals and/or inguinal adenopathy.

†This group includes: (1) proctitis and/or rectal stricture; (2) other anal lesions such as sinus, fistula, etc.; (3) involvement of colon.

‡This group includes cases presenting one of the following: (1) both genitoinguinal and rectal pathology, (2) generalized systemic manifestations, (3) laboratory infections, (4) other lesions not further classified.

An analysis of the data has brought out certain points of interest. In our hands the complement fixation test gave positive results with the sera from 98.6 per cent of 149 individuals in whom the Frei reaction and history or clinical signs were indicative of overt lymphogranulomatous infection; the two failures may have been due to a very low concentration of antibodies which could perhaps have been detected by making the conditions of testing more sensitive, or to the absence of antibodies in these particular serum specimens.

The titers of the positive sera ranged from 6 to 1,920 and greater. The frequency with which various titers were encountered was as follows:

		TITER
32	(21.8 per cent)	6 to 15*
40	(27.2 per cent)	30 to 60
42	(28.5 per cent)	120 to 240
33	(22.4 per cent)	480 or greater

The data have been examined in an attempt to ascertain the factors influencing the complement-fixing titers obtained.

1. *Activity Status:* Usually, but not invariably, the sera of patients with clinically active lesions showed higher titers than the sera of patients with healed lesions.<sup>22</sup>

2. *Intensity of Frei Reaction:* The sera of individuals who yielded a large cutaneous response usually showed higher titers than those from persons whose Frei reactions were weak (see also Grace and Rake<sup>22</sup>). In some instances, however, patients with antibodies of

\*It should be recalled that all figures concerning titer are given at threefold the usual designation in the literature. Thus most authors would give these titers as from 2 to 5.

low titer (6 to 15) gave skin reactions of greater intensity and extent than other persons whose serum titer was tenfold greater.

3. *Duration of Infection:* In recent infections the titers ranged from 6 to 960; where the disease had been in progress for three to eight years the titers observed were from 60 to 960. A patient with infection of ten years' standing was found to have a titer of 30; another whose disease dated back fifteen years showed a titer of 90, while the serum of a third individual twenty years after the onset of inguinal adenopathy showed a titer of 6. Thus the duration of infection was not necessarily reflected in the titer.

4. *Location of Lesions:* No obvious disparity in regard to antibody levels was noted between infections characterized by inguinal adenopathy and/or genital lesions and those involving the posterior regions of the bowel.

5. *Sex:* In the respective sexes the serum titers were distributed as follows:

TITER	WOMEN	MEN
6 to 15	8	24
30 to 60	6	25
120 to 240	15	24
480 or greater	4	8

While these figures indicate that titers of 120 or greater were more common in women, the number of cases involved is too small to be statistically significant.

6. *Race:* The titers in whites and Negroes were closely parallel, that is:

TITER	WHITES	NEGROES
6 to 15	19	13
30 to 60	16	15
120 to 240	21	18
480 or greater	7	5

7. *Concomitant Venereal Infection:* The presence of other venereal diseases did not noticeably affect the lymphogranuloma complement-fixing titer. It is noteworthy that in the group with rectal lesions (chiefly stricture and/or proctitis) thirty-five had no history or evidence of other venereal disease, twenty had signs, serologic evidence, or history of syphilis, and one had a history of gonorrhea. In the genitoinguinal group, eight had no evidence of other venereal disease, nine had a history of syphilis, and three had other venereal infections.

*Discussion:* It should be emphasized that in developing the complement fixation test with lymphogranuloma antigen, the results and technique were adjusted in an effort to set up conditions which would permit the detection of antibody in weakly reactive lymphogranulomatous sera. With the method adopted, specific fixation was obtained with the sera of 98.6 per cent of 149 clinically lymphogranulomatous, Frei-positive patients. In eleven of these the titer was only 6, and if the lowest initial dilution of serum tested had been 1:5 instead of 1:2, the demonstration of antibody would have failed. The importance of employing low as well as high dilutions of serum is thus evident, although Dulaney and Packer,<sup>23</sup> who in 97 per cent of their series of thirty-three clinically lymphogranulomatous patients obtained fixation with Lygranum in initial serum dilutions of 1:40 or greater, feel that a 1:20 dilution (corresponding to our titer of 60) should be considered as the lower limit for good serologic evidence of infection with the causative virus.

Several other workers have reported the results of complement fixation studies with Lygranum. Using a method similar to ours, Levine, Holder, and Bullowa<sup>21</sup> obtained fixation



with the sera of each of eleven known lymphogranulomatous patients and with the sera of eighteen other patients without overt lymphogranuloma venereum who yielded positive or doubtful-Frei tests. Blair<sup>25</sup> similarly obtained fixation with the sera of four patients showing clinically characteristic lymphogranuloma and thirteen other patients without history of the disease who had positive Frei reactions. A less favorable report was published by Knott and co-workers<sup>26</sup> who obtained positive tests by complement fixation in 75 per cent of a group of thirty-one patients diagnosed clinically as lymphogranulomatous. From their data it is doubtful whether all of the cases in the group represented true lymphogranulomatous infection. Only 84 per cent of their patients gave positive Frei tests and 45 per cent showed positive Ito-Reenstierna tests; in some of the patients the duration of the lesions was only three days. Also contributory to the failure of Knott and associates to obtain a higher percentage of positive results may be the fact that in their serologic tests complement was used at 1:10 dilution, a concentration two to three times greater than that which we employed, which might well reduce the sensitivity of the test so that it would fail to detect weakly reactive sera such as we found in 7.4 per cent of our lymphogranulomatous series.

Howard and co-workers<sup>27</sup> tested seventy-six patients with positive Frei reactions; the sera of seventeen gave negative complement fixation tests. The authors state that in this group ". . . in most instances there was a history suggesting infection with lymphogranuloma venereum in the remote past . . . there was no clinical evidence of infection at the time the tests were made; skin sensitivity alone remained." Unfortunately the details of the serologic technique employed in their study are not stated, making it difficult to evaluate the sensitivity of the method.

Recently Florman<sup>28</sup> has published the results of a series of tests with Lygranum antigen, employing a modified micro-Kolmer hemolytic system. His data indicate that this method was relatively insensitive for detecting weakly reacting sera, since in a series of parallel determinations on twelve sera he obtained nine positives using the method we have recommended, while only seven showed fixation when tested by his routine technique. Furthermore, in a group of eighteen patients with positive or doubtful Frei tests he obtained only eight positives by complement fixation, a far smaller proportion than that obtained by any of the others who have compared the cutaneous and serologic tests.

2. *Correlation of Complement Fixation Titer and Serum Protein Levels in Persons With Lymphogranuloma.*—Nicolas-Favre disease is frequently accompanied by the development of hyperglobulinemia and it has been suggested<sup>29</sup> that this might be due to the increased production of specific antibodies. So far no direct experimental evidence has been obtained in support of the hypothesis, since the sera of lymphogranulomatous patients usually exhibit only slight virus-neutralizing properties which make quantitative evaluation difficult. It is conceivable, however, that the increased globulin values seen in lymphogranuloma reflect the production of considerable amounts of antibody which react in the complement fixation test but are not effective in virus neutralization. An opportunity to examine the problem from this angle was afforded by Dr. Marion Howard, who furnished a number of such sera together with data on their values for globulin and their albumin: globulin ratios. Table II summarizes the results of our complement fixation tests on a group of sera which were tested within a few months of their collection from the patients, together with Dr. Howard's data on the protein values. We have not included the data on several other sera which were tested after two or more years' storage in the refrigerator.

TABLE II. LYGRANUM COMPLEMENT FIXATION TITERS AND PROTEIN VALUES OF LYMPHOGRANULOMATOUS SERA

PATIENT	COMPLEMENT FIXATION TITER	GLOBULIN	ALBUMIN: GLOBULIN
L. V.	Neg.	1.98	2.36
L. C.	6	2.37	1.8
L. DeL.	6	2.74	1.56
L. F.	6	2.72	1.6
W. C.	15	2.42	1.43
C. H.	15	2.55	1.8
F. G.	15	2.16	1.8
W. P.	15	2.68	1.6
H. M.	30	3.26	1.39
E. R.	30	3.51	1.20
W. M.	60	2.45	2.0
J. S.	60	2.89	1.52
M. M.	120	3.15	1.31
A. F.	120	4.19	0.9
A. C.	240	5.25	0.64
F. S.	960	5.42	0.72

It was found that the globulin values showed little deviation from the normal range in those patients whose sera had only low complement-fixing titers (6 to 15), whereas in six of eight patients with serum titers of 30 or greater, the globulin content was above 3 Gm. per 100 ml. serum; the maximum globulin values were obtained with sera which showed high complement-fixing activity. Our limited data, therefore, indicate positive correlation between elevation of the serum globulin and the titer of complement-fixing antibody versus lymphogranuloma antigen in patients with Nicolas-Favre disease.

3. *Tests on Sera from Persons Infected with Certain Viruses Related to the Agent of Lymphogranuloma Venereum.*—Data previously published<sup>12</sup> showed that the sera of patients with trachoma fixed complement specifically in the presence of lymphogranuloma virus antigens. We have now had the opportunity, through Dr. Phillips Thygeson, to test sera from thirty trachomatous individuals. Five of the sera were anticomplementary due to prolonged storage before testing; another patient's serum gave nonspecific fixation with normal yolk sac control material. Twenty-one of the remaining twenty-four sera fixed complement specifically in the presence of lymphogranuloma antigens prepared from elementary body suspensions sedimented by high-speed centrifugation, or the soluble antigen obtained in Seitz filtrates, or both. The titers were usually low and more readily demonstrable by fixation overnight in the refrigerator. Complement fixation was demonstrable with the sera from three of four patients who had shown clinical healing under the influence of sulfonamide therapy. Despite the presence of complement-fixing antibodies in their sera the Frei test was uniformly negative whenever performed on trachomatous patients.

Sera from seven patients with psittacosis were obtained through Dr. K. F. Meyer,\* who also furnished the results of complement fixation tests carried out with psittacosis antigen in his laboratory. Each of the sera showed strong complement fixation with Lygranum antigen, our titers ranging from 120 to 1920.<sup>13</sup>

\*The Hooper Foundation, San Francisco, Calif.

Complement fixation with both lymphogranuloma and psittacosis antigens was also demonstrable with each of the sera from seven patients with virus pneumonitis which was presumed or proved due to an agent related to the meningopneumonitis virus of Francis and Magill.<sup>30</sup> Six of these sera<sup>13</sup> were obtained from Dr. M. D. Eaton;\* the seventh specimen was furnished by Dr. John H. Dingle.† These seven sera gave titers ranging from 6 to 60 when tested with Lygranum.

Through Dr. Thomas P. Magill‡ sera were also obtained from eleven patients with "atypical pneumonia" where there was no evidence to incriminate as etiologic agents any members of the psittacosis-lymphogranuloma group of viruses. None of these sera gave fixation with Lygranum.

*Discussion:* Complement fixation tests on the sera of patients infected with various viruses of the psittacosis-lymphogranuloma group have shown that these agents must share a common antigenic factor which stimulates the production of antibodies responsible for cross reactions within the group. The definite, albeit weak, reactions we observed when trachomatous sera were tested with lymphogranuloma antigen served to re-emphasize the close biologic relationship between these two etiologic agents which was previously predicated on the basis of striking similarities in morphology, staining, intracytoplasmic growth, and other characteristics. Unfortunately, trachoma viral antigen was not available for reciprocal tests with lymphogranulomatous sera.

Cross reactions of high titer have been demonstrated repeatedly<sup>13, 14b, 15, 17c, 24</sup> for human psittacotic and lymphogranulomatous sera not only when they are tested with antigens from either of these viruses, but also in the presence of antigens derived from the viruses of meningopneumonitis<sup>30</sup> and mouse pneumonitis.<sup>31</sup> Differences have been noted in the titers of individual sera versus the respective viral antigens used for testing, but these may have been due, in part at least, to varying potency of the antigens and to differences in the techniques of testing employed in the several laboratories. Smadel, Wertman, and Reagan<sup>14b</sup> have shown in this connection that the sera of patients with either psittacosis or lymphogranuloma venereum usually give complement fixation to approximately the same titer when tested simultaneously with standardized antigen from the heterologous or homologous virus.

The sera of various animal species infected or immunized with members of the psittacosis-lymphogranuloma group of agents show differing degrees of specificity in complement fixation tests with the several viral antigens. The situation is not entirely clear in regard to the serologic response of pigeons, which often acquire ornithosis naturally.<sup>32a</sup> Although the sera of birds convalescent from enzootic or epizootic ornithosis have been reported not to give fixation with Lygranum,<sup>14b, 32b</sup> two of three pigeon sera sent us by Dr. K. F. Meyer did, in fact, give strong fixation with this antigen (titers 120 and 480, respectively).<sup>13</sup> Further, Beck and co-workers<sup>15b</sup> found in three of four pigeons infected with the S-F strain of human pneumonitis virus a slight rise in antibodies to lymphogranuloma venereum antigen.

Several serologic studies have been carried out among persons with pneumonitis or atypical pneumonia where no direct contact with infected birds was known. Eaton and colleagues investigated two groups of such cases. In one small series an agent related to

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the viruses of meningopneumonitis and psittacosis was incriminated as etiologic agent.<sup>32a</sup> These patients all showed antibodies reacting indiscriminately with psittacosis, meningopneumonitis, lymphogranuloma, and mouse pneumonitis antigens; in several instances the development of a rise in titer for these antigens during the course of the illness was demonstrated.<sup>14a</sup> It may be noted here that in the case of another member of the psittacosis-lymphogranuloma group of viruses, namely, that causing an epidemic of severe pneumonitis in the bayou region of Louisiana, serum specimens from a few patients who had been infected showed fixation not only with antigen from the specific etiologic agent, but also with preparations from other viruses in the related group.<sup>32b</sup> In a second, larger series of cases of pneumonitis of unknown etiology which were studied by Eaton and Corey the sera of roughly 10 per cent of the patients showed a fourfold or greater increase in titer versus these antigens during convalescence when compared with samples collected during the acute phase.<sup>11a</sup> Smadel<sup>14c</sup> examined the sera of forty-five cases of atypical pneumonia drawn from several large urban centers of eastern United States. Ten cases were believed to be due to psittacosis; five other cases were considered on the basis of complement fixation tests possibly to be of psittacotic origin, but the sera of these patients showed no significant increase in titer during the course of recovery from the disease.

Dingle and associates,<sup>34</sup> in their extensive studies on atypical pneumonia, encountered six patients with respiratory disease from whom strains of virus resembling the agent of meningopneumonitis were isolated in the laboratory of Dr. Thomas Francis, Jr.\* During the course of illness none of these six showed any rise in the titer of antibodies reacting with the agent of meningopneumonitis, whereas five other patients with respiratory infections, of whom only one showed atypical pneumonia, developed a fourfold or greater increase in serum titer during the course of illness. Complement fixation tests versus meningopneumonitis antigen were then carried out on the sera of a large group of well soldiers and patients with various respiratory infections. While positive reactions were only infrequently encountered among the sera from white donors, there was an appreciable concentration of positive reactions among the Negro group, irrespective of history of respiratory disease or evidence of atypical pneumonia. Since the Negroes were drawn chiefly from the South, where the incidence of lymphogranuloma is known to be high, it was suspected that the incidence of positive reactors among them might be due to crossreactions resulting from infection with lymphogranuloma virus.

Thomas and co-workers<sup>35</sup> made the observation that in persons with atypical pneumonia of unknown etiology sera taken during the convalescent phase frequently showed considerable complement-fixing activity versus crude mouse lung suspensions infected with any of several dissimilar viruses, including members of the psittacosis-lymphogranuloma group. These sera also reacted, although to a lesser extent, with similarly prepared suspensions of normal lungs from human beings and from mice and other laboratory animals, and with normal chick yolk sac. Acute phase sera from the same patients showed much less reactivity. It is interesting that the convalescent sera did not react with antigen of psittacosis virus propagated in tissue culture. The capacity to react with dissimilar antigens could be reduced by merely heating the sera to 60 to 65° C. or by absorption with normal mouse lung. The antigenic component responsible for fixation with atypical pneumonia sera was associated with large particles contained in the crude lung suspensions, since it could be reduced by centrifugation.

From the foregoing we conclude that: (1) antibodies reacting with Lygranum are usually demonstrable in the sera of nonlymphogranulomatous persons who are known or presumed to be infected with any one of the several other members of the group of related viruses; (2) in sera from cases of atypical pneumonia of unknown etiology, fixation versus lymphogranuloma virus is ordinarily lacking if infection with viruses of the psittacosis-lymphogranuloma group can be excluded or is highly unlikely; (3) in some cases of atypical

\*University of Michigan.

pneumonia there develop during convalescence substances which react more or less nonspecifically with certain tissue components from uninfected or virus-infected lung or other tissue. These latter reactions can frequently be distinguished from specific fixation occurring with virus antigens through the use of control antigens prepared with normal tissue; they can also be reduced or eliminated by preliminary heating of the sera to 60 or 65° C., as we have done.

4. *Tests on Persons With Evidence of Syphilis.*—Complement fixation tests with Lygranum were performed on the sera of 131 individuals having evidence of syphilis. Some of the sera were specimens collected for routine premarital examinations; a large proportion was collected from patients attending venereal disease clinics. Various stages of syphilis were represented including latent, primary, secondary, and neurosyphilis. In thirty-four of the patients the syphilitic infection was congenital. Table III summarizes the results of the tests.

TABLE III. LYGRANUM COMPLEMENT FIXATION TESTS ON SERA OF PERSONS WITH EVIDENCE OF SYPHILIS

SOURCE OF INFECTION	NUMBER TESTED	LYGRANUM COMPLEMENT FIXATION		
		POSITIVE	NEGATIVE	NONSPECIFIC
Congenital	34	2*	32†	0
Acquired	97	59	30	8

\*These patients were Frei-positive, married, white women.

†Thirty of these patients were also Frei tested; all gave negative reactions.

In sharp contrast to the low incidence of positive serologic or cutaneous reactions with Lygranum among those with congenital syphilis<sup>36</sup> are the findings in the group of individuals whose syphilitic infection was otherwise acquired. Forty-five of these ninety-seven were also given Frei tests. The findings are given in Table IV.

TABLE IV

LYGRANUM COMPLEMENT FIXATION	FREI REACTION	NUMBER IN GROUP
Pos.	Pos.	10
Pos.	Doubt.	3
Pos.	Neg.	18
Neg.	Neg.	7
Nonsp.	Neg.	6
Neg.	Nonsp.	1

Thirteen (29 per cent) of the forty-five persons with syphilis of noncongenital origin yielded positive or doubtful Frei tests; the sera of all thirteen fixed complement specifically in the presence of Lygranum. These individuals included five white women, five Negro men, and three Negro women; from none of them was a history of overt lymphogranuloma elicited and on examination they were asymptomatic. An additional 38 per cent of the group of forty-five gave positive complement fixation with Lygranum despite negative Frei tests.

It may be noted that the sera of eight patients with acquired syphilis showed equal fixation with normal yolk sac material and with Lygranum antigen. The significance of the various findings in the patients with syphilis as

well as in those with other genitoinfectious diseases will be discussed following the presentation of the experimental data in the next section.

5. *Tests on Sera of Persons With Genitoinfectious Diseases Other Than Syphilis.*—Complement fixation tests with lymphogranuloma antigen were performed on a limited number of sera from persons in this category, the results being summarized in Table V.

TABLE V. LYGRANUM COMPLEMENT FIXATION TESTS ON SERA OF PERSONS WITH GENITO-INFECTIOUS DISEASES OTHER THAN SYPHILIS

DISEASE	NUMBER TESTED	NUMBER POSITIVE
Gonorrhea		
Vaginitis of childhood	5	0
Venereally acquired	19	9
Chancroid	6	3

It will be seen that roughly the same proportion of sera from persons with venereally acquired gonorrhea or chancroid were positive versus lymphogranuloma antigen as was found for the group of syphilitic patients with acquired infection. Similarly, the failure to obtain fixation with the sera of children showing gonococcal vaginitis corresponds with the findings in the congenital syphilitic group. Frei tests were performed on the patients with chancroid; one of the three whose sera showed fixation also manifested a positive cutaneous response.

*Discussion:* The foregoing findings among the patients with syphilis and other genitoinfectious diseases are of considerable interest. Although 61 per cent of the group of ninety-seven with acquired syphilis gave positive serologic reactions with Lygranum, many of the members of the group tested were patients coming to venereal disease clinics, and we do not consider that this figure necessarily reflects accurately the incidence which one should expect to encounter elsewhere in large-scale tests on the general population. As reported previously,<sup>11b</sup> among the Negroes of the group the incidence was considerably higher in respect to positive reactions with Lygranum in both the cutaneous and complement fixation tests. In the work of Dulaney and Packer<sup>23</sup> it was noted similarly that among twenty-two Negro children with congenital syphilis no positive complement fixation reactions with Lygranum were obtained, whereas with sera from adults with neurosyphilis this serologic test was frequently positive and again the incidence of such reactions was definitely higher among Negroes than among whites.

Knott and colleagues<sup>26</sup> have also reported the study of a group of patients with early clinical syphilis, chiefly Negroes, attending the venereal disease clinic at the Johns Hopkins Hospital. Forty-three of the seventy-eight cases were given cutaneous tests with human Frei antigen; thirteen responded with positive or doubtful reactions. This is in surprisingly close agreement with our findings of thirteen positive or doubtful cutaneous reactions among forty-five syphilitic patients tested with Lygranum. Twenty-three of seventy-eight in the Johns Hopkins series gave complement fixation with Lygranum only; an additional thirty-four showed fixation with normal yolk sac material as well as with Lygranum. The Baltimore workers regarded as positive results all complement fixation reactions occurring with Lygranum, even when the same serum reacted equally with normal yolk sac control material. We,

on the contrary, have emphasized from the outset the difficulty of reaching any valid decision as to the specificity of a Lygranum complement fixation test when there is equal degree of fixation with the normal yolk sac control. We have considered the combined reactions as nonspecific since they are in most instances due to yolk material not removed during the preparation of the viral antigen; it is known, for example, that yolk lipids enhance the sensitivity of serologic reactions used in the diagnosis of syphilis. Our own data have included as positive reactions only those where fixation with lymphogranuloma virus antigen was marked and the reaction with yolk sac control material was entirely lacking or minimal. More recently it has been shown<sup>17c</sup> that many of the nonspecific reactions obtained with syphilitic sera can be eliminated through the use of antigen preparations treated with phenol and/or boiling.

Knott and co-workers<sup>26</sup> found that for syphilitic sera showing fixation with normal yolk sac material as well as with Lygranum antigen, absorption with Eagle flocculating antigen in many instances removed the reactivity versus both normal yolk sac control and viral antigens. A similar reduction in some cases followed antisyphilitic therapy; in other patients whose sera showed reactivity to both Lygranum and normal yolk sac control, antisyphilitic treatment was followed by a reduction in titer versus the control antigen while the titer versus Lygranum persisted. These results lend emphasis to our views as to the difficulty of assessing the specificity of fixation with Lygranum when the reaction with normal yolk sac material is also positive. Further, in certain syphilitic patients showing serologic reactivity versus Lygranum but not versus the control material, Knott and associates<sup>26</sup> observed that antisyphilitic treatment did not reduce the reactivity to Lygranum, nor did absorption with Eagle flocculating antigen remove the property from the serum. (See also<sup>11b, 23</sup>.) They believed that in these latter cases at least part of the original reaction was due to specific antibody versus the virus, indicating lymphogranulomatous infection. They suggested that the results of Lygranum complement fixation in syphilitic patients could be evaluated best by repeated tests during the course of antisyphilitic treatment.

The Baltimore investigators were doubtful of the specificity of complement fixation with Lygranum because of the large number of positive tests they obtained with sera of patients giving no history or signs of overt lymphogranuloma. Koteen<sup>27</sup> has pointed out, however, that factual data are difficult to obtain from Negroes coming to a venereal disease clinic because these patients are prone to overlook small and painless lesions; negative histories therefore must be regarded with suspicion. Despite this, it may be noted that among thirty-four syphilitic patients of whom specific inquiry was made by Knott and colleagues, sixteen gave a history of previous genital lesions which might have been lymphogranulomatous.

Beeson and Miller<sup>28</sup> have recently made an important contribution to our understanding of the problem. They carried out Kahn and Lygranum complement fixation tests on 879 sera from an unselected group of white and Negro patients attending outpatient clinics at the Grady Hospital in Atlanta, Ga. The results were analyzed according to race, age, and sex. Among adult Negroes, the sera of approximately 40 per cent gave positive fixation with Lygranum and the sera of 32 per cent yielded positive Kahn tests. Among adult whites, the sera of 12 per cent gave positive fixation with Lygranum and 9 per cent gave positive Kahn tests. The age distribution of donors with positive serologic reactions followed much the same pattern for the Kahn test as for fixation with Lygranum, with a sharp rise in incidence after 14 years of age. The number of persons giving a positive test for one disease and a negative test for the other was in all groups greater than the number giving positive tests for both diseases. Furthermore, while 60 per cent of Negroes, both men and women, with positive Kahn gave positive Lygranum fixation, among the white patients with syphilis the sera of only 18 per cent of the men and 21 per cent of the women gave fixation with Lygranum, a racial difference which would not be expected if syphilis were a frequent cause of false positive serologic tests for lymphogranuloma. Beeson and Miller considered it probable that the high incidence of positive tests with Lygranum in individuals with syphilis and other venereal diseases is due to actual associated lymphogranuloma venereum infection, even in the absence of clinical findings.

From the data and discussion just presented, it appears that patients with evidence of another venereally acquired genitoinfectious disease (whether this be syphilis, chaneroid, or gonorrhea) frequently show positive cutaneous and/or serologic reactivity versus Lygranum. This is in sharp contrast to the low incidence of reactions observed in patients who have acquired the same diseases by nonvenereal means. We do not believe that the high incidence of serologic reactions in persons with diseases of such diverse etiology is due to nonspecific factors but rather attribute the great bulk of the reactions to an antecedent or concomitant infection with lymphogranuloma virus even though overt clinical manifestations of the latter may be few or lacking unless very careful search is made. A small proportion of the serologic reactions observed among these patients may, however, reflect a previous infection with some other member of the psittacosis-lymphogranuloma group.

6. *Tests on Sera From Persons With Miscellaneous Respiratory and Non-respiratory Infections.*—The data presented in the preceding sections have shown that complement fixation with Lygranum was exhibited by a high percentage of sera from (1) persons infected with agents of the lymphogranuloma-psittacosis group and (2) those with various infections of venereal origin. It seemed desirable to examine the sera of persons suffering from infections with agents unrelated to lymphogranuloma virus in which the likelihood of venereal acquisition was slight in order to determine the incidence of positive reactions in a contrasting group.

Among sixteen patients with clinically active tuberculosis, chiefly pulmonary, the sera of only two were positive versus Lygranum. One of the positive specimens was from a patient with a history of several previous attacks of gonorrhea; nothing was known about the venereal history of the second patient. The remaining donors, whose sera yielded negative results, included two with tuberculous lesions of the perianal region and genitals, respectively.

Among nine sera from patients with one of a variety of nontuberculous respiratory infections, none gave fixation with Lygranum. Similarly, in the group of eight patients listed as having various nonrespiratory infections, the complement fixation tests with Lygranum were uniformly negative (Table VI).

It is evident from the foregoing that neither the mere presence of an acute or chronic infectious process nor its localization in a site frequently affected by members of the psittacosis-lymphogranuloma group of viruses can be responsible for the findings reported in Sections 1, 3, 4, and 5 of this paper. Our failure to obtain complement fixation with lymphogranuloma antigen among the sera from the group of patients with assorted respiratory infections (Table VI), including atypical pneumonia of origin unrelated to the psittacosis-lymphogranuloma group (see Section 3), is in contrast to the findings of Florman<sup>25</sup> and Dulaney and Packer<sup>23</sup> who did obtain reactions with Lygranum C. F. in tests on sera from patients with various respiratory infections. It is possible that the difference in results may be due to the fact that we employed antigens in which the virus was separated from the bulk of host tissue materials, whereas the commercially available Lygranum used by these workers was



less refined. As pointed out previously, Thomas and co-workers<sup>35</sup> have shown that the sera of patients with atypical pneumonia have a tendency to fix complement in the presence of relatively crude suspensions of a variety of animal tissues.

TABLE VI. LYGRANUM COMPLEMENT FIXATION TESTS ON SERA FROM PERSONS WITH CERTAIN NONVENEREAL INFECTIONS

PATIENT	CLINICAL CONDITION	COMPLEMENT FIXATION TITER
Wh.	Upper respiratory infection (Acute)	Neg.
P. B.	Upper respiratory infection (Convalescent)	Neg.
C.	Upper respiratory infection (Convalescent)	Neg.
H.	Pneumonia (Pn. type IX) (Acute)	Neg.
D.	Pneumonia (Convalescent)	Neg.
McK.	Pneumonia (Convalescent)	Neg.
M. K.	Pneumonia; pleurisy	Neg.
O. R.	Empyema	Neg.
Z.	Lung Abscess	Neg.
M.	Septicemia ( <i>Salmonella suispestifer</i> )	Neg.
F.	Septicemia ( <i>Streptococcus hemolyticus</i> )	Neg.
P.	Erysipelas	Neg.
S.	Osteomyelitis	<15
J. B.	Lymphocytic choriomeningitis	<15
A.	? Meningitis	Neg.
T.	? <i>Escherichia coli</i> sepsis	Neg.
W.	? Dysentery	Neg.

\*Titers reported < signify that the specimens were anticomplementary at lower dilutions tested and negative at the titer indicated.

7. *Tests on Sera From Persons With Certain Organic Diseases.*—For additional information as to the range of specificity of the complement fixation obtained with Lygranum, tests were made on the sera of patients with evidence of neoplasia or cardiovascular, renal, or hepatic damage.

In the group of fourteen patients with neoplasia, the following manifestations were included: carcinoma of stomach, 2; carcinoma of sigmoid colon, 1; carcinoma of rectum, 2; papilloma of rectum, 1; rectal polyp, 2; carcinoma of pancreas, 2; carcinoma of gall bladder, 1; carcinoma of prostate, 1; lymphoma, 1; lymphosarcoma, 1. On examination one serum was anticomplementary to high titer; two other sera were anticomplementary at initial dilutions of 1:15 and 1:5, respectively, but at higher dilutions gave no evidence of specific fixation. The only positive reactions (titers 15 and 6) were obtained with sera from two men who exhibited rectal carcinoma and rectal polyp, respectively.

The fifteen patients with evidence of cardiovascular damage included individuals with congenital heart disease, various degrees of arteriosclerosis, hypertension, rheumatic heart disease, or subacute bacterial endocarditis. Only one serum, taken from a patient with rheumatic heart disease who also had a history of thyroidectomy, diabetes, and postoperative tetanus, was positive and this solely at the lowest dilution tested (titer 6).

Among ten patients with evidence of kidney damage, including glomerulonephritis, pyelonephritis, nephrosis, horseshoe kidney, pyelitis, staghorn calculus, or sulf- and methemoglobinuria, none of the sera showed specific fixation with Lygranum.

On the other hand, in a group of twenty patients with liver damage, in most of whom a diagnosis of cirrhosis was made, the sera of eight individuals gave definite fixation with titers ranging from 6 to 60 (Table VII).

TABLE VII. LYGRANUM COMPLEMENT FIXATION TESTS ON SERA FROM PERSONS WITH EVIDENCE OF LIVER DAMAGE

PATIENT	CLINICAL CONDITION	EVIDENCE OF VENEREAL DISEASE	FREE REACTION	COMPLIMENT FIXATION TITER
C.	Cirrhosis	Neg.	Neg.	15
N. C.	Cirrhosis	Neg.	Neg.	60
D.	Cirrhosis	—	—	Neg.
G.	Cirrhosis	—	—	Neg.
J.	Cirrhosis	—	—	Neg.
K.	Cirrhosis	Neg.	—	15
R. L.	Cirrhosis	Neg.	Neg.	15
P.	Cirrhosis	—	—	<15
E. S.	Cirrhosis	Neg.	—	Neg.
S.	Cirrhosis	—	—	15
T. B.	Cirrhosis; arteriosclerosis; heart disease	Neg.	—	15
W. C.	Cirrhosis; nontoxic thyroid adenoma	Neg.	—	Neg.
G.	Cirrhosis; pneumonia (Pn. type VII)	Neg.	—	15
C. M.	Cirrhosis; generalized arteriosclerosis; mild diabetes	Neg.	Neg.	6
E. O.	Cirrhosis; bronchopneumonia	Syph.	Neg.	<15
R. C.	? Cirrhosis; jaundice	Neg.	—	15
H. G.	? Acute yellow atrophy or cirrhosis; thyrotoxicosis	—	—	30
R. G.	Miliary cirrhosis	Neg.	Neg.	<15
J. B. F.	Postinfectious streptococcal hepatitis	Neg.	Neg.	<15
R. L. K.	Hepatitis (? posttyphoidal)	Neg.	—	Neg.

*Discussion:* The examination of sera from persons with cardiovascular or renal disease or neoplasia offered little or no evidence that complement-fixing antibodies versus Lygranum are developed as a result of any of these conditions per se. The positive reactions obtained with an appreciable proportion of sera from individuals showing signs of liver damage and with no overt evidence of lymphogranulomatous infection were at first rather surprising. The nature of these reactions is still not clear, and we are not in a position to draw any definite conclusion as to the antecedent history of the patients which might be responsible for this behavior of the sera. It is well known that increased globulin and decreased albumin levels may be encountered in hepatic cirrhosis, so it might be assumed that the complement-fixing reactivity of the sera with Lygranum is due to physiologic changes which are of an immunologically non-specific character. There is also the possibility that some of the positive complement fixation reactions in this group of patients may indicate infection with some, perhaps as yet unrecognized, virus related antigenically to the members of the psittacosis-lymphogranuloma group of agents which may result in liver damage simulating that of cirrhosis.

Certain recent observations<sup>39</sup> are of interest in this connection. It has been possible to show that when mice are inoculated intravenously with a sufficiently large dose of the agent of mouse pneumonitis (Nigg), most of the animals will die within three or four hours. With smaller doses, however, life may be prolonged for as long as a week or more. Many of these mice become bloated and at autopsy show generalized edema, ascites, and pleural effusion as well as microscopic changes of early cirrhosis in the liver. Other observations given in the afore-mentioned studies<sup>39</sup> and elsewhere<sup>40</sup> have shown that all of the toxins of the psittacosis-lymphogranuloma group of agents cause damage to the liver cells in particular. This would support the view that some cases of human cirrhosis may be due, in part at least, to infection with an agent belonging to this group. (See also <sup>41, 42</sup>) Since most instances of cirrhosis, however, are undoubtedly due to other causes, the negative serologic findings with the majority of cirrhotic sera are readily explained.

8. *Tests on Sera From Laboratory Personnel.*—As part of our control series we have tested the sera of thirty-two laboratory workers, chiefly from New Brunswick personnel; the results are summarized in Table VIII.

TABLE VIII. LYGRANUM COMPLEMENT FIXATION TESTS ON SERA OF LABORATORY PERSONNEL

HISTORY OF LYMPHOGRANULOMA VENEREUM	NUMBER OF PERSONS TESTED	COMPLEMENT FIXATION			FREI REACTION POS.
		POS.	NEG.	NONSP.	
Negative	28	0	27	1	—
Equivocal	1	1	0	0	1
Laboratory infection	5	5*	0	0	5

\*The sera of two of these workers, taken before the known infection, had been negative.

Sera from five cases of undoubted laboratory infection were examined. One was from a worker in another laboratory who developed lymphogranulomatous ophthalmitis<sup>43</sup>; his serum gave definite fixation with Lygranum antigen to a titer of 60. Two New Brunswick workers contracted the disease during the course of intensive animal experimentation with highly infectious material.<sup>19</sup> In each, the complement fixation titer of the serum rose markedly and subsided only very gradually despite the rapid alleviation of clinical signs and symptoms as a result of sulfathiazole therapy. The strongly positive Frei reactions which both workers showed in the early stages of their illness diminished in intensity during the year subsequent to infection, as shown by repeated tests during this period. Still another member of the New Brunswick personnel who was exposed to very infectious material during the frequent handling of glassware containing lymphogranuloma virus developed fever, general malaise, and pain in the region of the left shoulder due presumably to local adenopathy. In this case also, the Frei and complement fixation tests were strongly positive (serum titer 480) and administration of sulfathiazole resulted in the clearing of the general and local malaise. In the fifth individual the presence of lymphogranuloma was undiagnosed until routine complement fixation tests on the laboratory personnel revealed that his serum was strongly positive versus Lygranum. He had assisted in the care of lymphogranulomatous mice for several months and was discovered to have a history compatible with that of recent accidental infection including the development of minor herpetiform lesions on the back of one hand together with general malaise, slight fever, and headache over a

period of several weeks. His condition showed marked amelioration following treatment with sulfathiazole per os.

*Discussion:* From the foregoing observations of laboratory workers we conclude that the sera of persons in whom the likelihood of infection with agents of the lymphogranuloma-psittacosis group is minimal rarely contain complement-fixing antibodies versus Lygranum antigen. Knott and co-workers<sup>35</sup> obtained similar results on testing twenty white members of the hospital staff personnel as controls in their study; all of these gave negative reactions to the cutaneous and serologic tests with Lygranum.

On the other hand, cases of laboratory infection with lymphogranuloma virus, even though acquired by routes and modes other than those usual for this disease, not only become Frei positive, but also develop complement-fixing antibodies early in the course of illness, sometimes within a week after the onset; these may persist in the serum for over two years, although with the cessation of clinical activity and the possible elimination of the incitant as a result of intensive therapy with drugs of the sulfonamide group, the antibody titer tends to diminish and the Frei reaction may become completely negative again. In patients with early manifestations of the naturally acquired disease, similar reversal of the Frei reaction as a result of vigorous sulfonamide chemotherapy has been reported by others,<sup>3d, 37, 44</sup> although it does not commonly occur.

It may be pointed out that, as in the natural disease, laboratory infections with the virus of lymphogranuloma may vary widely in clinical manifestations. Some are mild<sup>45</sup> and would be unnoticed unless a Frei or complement fixation test were performed, while others may present signs of acute systemic disease.<sup>19</sup> It would seem a highly desirable precaution that individuals working intensively with the agent should perform complement fixation tests on their own sera at frequent intervals to check on the possibility of infection.

*9. Tests on Sera From Persons With Genitoinguinal or Lower Bowel Lesions of Questionable Origin.*—In the course of this study there was opportunity to investigate the value of the complement fixation test in affording evidence to confirm or exclude the diagnosis of probable infection with the agent of lymphogranuloma venereum in: (1) patients with genitoinguinal lesions compatible with those of early lymphogranuloma but in whom the Frei test was not definitely positive; (2) patients with genitoinguinal lesions of questionable origin but with positive Frei reaction; (3) patients with genitoinguinal lesions of questionable origin and negative Frei reaction. From Table IX it may be seen that in the small number of cases tested, complement fixation with Lygranum was regularly obtained with the sera of patients in the first two groups but with only certain sera from patients in the third group. In this latter group the positive results were obtained in those patients whose history and signs suggested the possibility of previous lymphogranulomatous infection.

In Table X are summarized the data on seventeen patients, each of whom presented one of a variety of lower bowel lesions of questionable origin. Two patients showed positive or doubtful Frei reactions, and the sera of both gave fixation with Lygranum. Among the remaining fifteen patients, the sera of only four yielded positive reactions. As might be expected, this incidence is con-

siderably lower than was observed with the sera of persons who showed bowel lesions regarded clinically as lymphogranulomatous and who also yielded positive Frei reactions (see Table I).

TABLE IX. LYGRANUM COMPLEMENT FIXATION TESTS ON SERA FROM PERSONS WITH GENITOINGUINAL LESIONS OF QUESTIONABLE ORIGIN

GROUP	PATIENT	RACE	SEX	LESIONS PRESENTED	EVIDENCE OF OTHER VENEREAL DISEASE	FREI REACTION	COMPLEMENT FIXATION TITER
a	C. A.	W	F	Inguinal adenopathy, recent	Neg.	Neg.	120
	J. G.	W	M	Inguinal adenopathy, recent plus penile lesion	Neg.	Neg.	6
	A. H.	W	M	Inguinal adenopathy, recent	Syph.	Doubt.	120
	R. L.	W	M	Inguinal adenopathy plus penile lesion	Ducrey test Pos.	Neg.	60
b	R. B.	W	F	Hard mass in pelvis; ? fibrotic lymph nodes	Neg.	Pos.	6
	C. B.	N	M	Mass in right seminal vesicle, reduced with sulfathiazole	Syph.	Pos.	6
	C. G.	W	M	Fibrosis of corpora cavernosa	Gonor.	Pos.	6
	M. J.	N	F	Bartholin's abscess	Syph.	Pos.	120
	J. L.	W	M	Balanitis	Neg.	Pos.	6
	W. W.	W	M	Nodule in scrotum	Neg.	Pos.	6
c	L. B.	W	F	"Spot on womb"	—	Neg.	Neg.
	H. L.	W	F	Acanthosis of vulva	Neg.	Neg.	<60
	V. M.	N	F	Ulcerating lesion of vulva; improved with sulfanilamide	Neg.	Neg.	15
	M. M.	W	M	Right inguinal scar and sinus; serotal swelling	Neg.	Neg.	Neg.
	O. S.	W	M	History of inguinal adenopathy; mass between rectum and peritoneum	Neg.	Neg.	6
	L. S.	Y	M	Old inguinal scars	Syph.	Neg.	6

TABLE X. LYGRANUM COMPLEMENT FIXATION TESTS ON SERA FROM PERSONS WITH LOWER BOWEL LESIONS OF QUESTIONABLE ORIGIN

PATIENT	RACE	SEX	CLINICAL CONDITION	FREI REACTION	COMPLEMENT FIXATION TITER
R. B.	W	M	Ulcerative colitis	Neg.	6
N.	—	—	Ulcerative colitis	—	Neg.
C. S.	W	M	Ulcerative colitis	—	Neg.
F. V.	W	M	Ulcerative colitis	Neg.	Neg.
C. E.	W	M	Stricture from hemorrhoidectomy	Neg.	Neg.
M. B.	W	M	Stricture from hemorrhoidectomy	Neg.	Neg.
H. G.	W	M	Rectal stricture	Neg.	Neg.
J. G.	W	M	Rectal stricture	Neg.	Neg.
W. B.	W	M	Pruritus ani	Neg.	Neg.
A. H.	W	M	Pruritus ani	Neg.	6
M. S.	W	F	Fissure in ano	Neg.	Neg.
H. W.	W	M	Perianal fissure	Neg.	120
E. L.	W	M	Fistula in ano	Neg.	6
C. M.	W	F	Rectal nodule	Neg.	Neg.
S. Y.	Y	M	Rectal polyp with histologic picture of lymphogranuloma	Doubt.	320
C. E.	W	M	? Carcinoma of rectum in 1932; no recurrence after abdominoperineal resection	Pos.	60
R. G.	W	F	Lymphocytic granuloma of rectum	Neg.	Neg.

*Discussion:* The limited data presented in this section indicate that the complement fixation test with *Lygranum* may be of definite aid in the establishment of an etiologic diagnosis in certain cases of genitoinguinal or lower bowel disease where the lack of a positive Frei reaction or the character of the lesions raises some doubt as to their lymphogranulomatous nature. In such cases repeatedly negative serologic tests would make infection with the agent of Nicolas-Favre disease seem highly unlikely, while positive tests are suggestive of an infection with some member of the lymphogranuloma-psittacosis group of agents and call for further study of the patient, including repeated Frei tests. It has been observed that the latter may subsequently become positive, thus confirming the earlier serologic findings.

10. *Tests on Sera From Persons With Urogenital Lesions of Questionable Origin.*—In Table XI are presented data concerning the findings in a group of

TABLE XI. LYGRANUM COMPLEMENT FIXATION TESTS ON SERA FROM PERSONS WITH UROGENITAL LESIONS OF QUESTIONABLE ORIGIN

PATIENT	RACE	SEX	CLINICAL CONDITION	EVIDENCE OF OTHER VENEREAL DISEASE	FREI REACTION	COMPLEMENT FIXATION TITER
N. D.	W	M	Urethritis	Neg.	Neg.	15
M. F.	W	M	Urethritis	Neg.	Neg.	Neg.
G.	W	M	Urethritis	Neg.	Pos.	30
R. S.	N	M	Urethritis	Neg.	Pos.	30
C. S.	N	M	Urethritis	Neg.	Pos.	6
H. S.	W	F	Urethritis and cystitis	Neg.	Neg.	6
R. T.	W	F	Urethritis and cystitis	Neg.	Pos.	15
F. W.	W	M	Urethral stricture	Neg.	Neg.	30
J. D.	W	M	Urethral stricture, prostatitis and periurethral abscess	Neg.	Doubt.	6
S. P.	W	M	Urethral stricture and prostatitis	Neg.	Neg.	Neg.
E. N.	W	M	Urethral stricture and carcinoma of prostate	Gonor.	Neg.	15
G.	W	M	Urethral fistula	Neg.	Neg.	15
M.	—	—	Prostatic hypertrophy	Syph.	—	Neg.
F. B.	W	M	Cystitis	Neg.	Neg.	Neg.
M. C.	W	M	Cystitis	Neg.	—	Neg.
F. G.	—	—	Cystitis	Neg.	Neg.	Neg.
A. G.	—	—	Cystitis	Neg.	—	Neg.
J. K.	W	M	Cystitis	Gonor.	Pos.	120
A. L.	W	F	Cystitis	Neg.	Neg.	Neg.
L. M.	W	F	Cystitis	Neg.	Neg.	Neg.
M. N.	W	F	Cystitis	Neg.	Neg.	15
O.	W	F	Cystitis	Neg.	Neg.	Neg.
L. P.	W	F	Cystitis	Neg.	Neg.	Neg.
S. P.	W	F	Cystitis	Neg.	Neg.	Neg.
P.	W	F	Cystitis	Neg.	Neg.	Neg.
J. P.	W	F	Cystitis	Neg.	Neg.	Neg.
E. R.	N	M	Cystitis	Neg.	Neg.	Neg.
I. R.	W	M	Cystitis	Neg.	Neg.	Neg.
J. S.	W	M	Cystitis	Gonor.	Pos.	15
M. S.	W	F	Cystitis	Neg.	Pos.	15
I. W.	W	F	Cystitis	Neg.	Doubt.	Neg.
L. W.	W	F	Cystitis	Neg.	Neg.	Neg.
W.	W	F	Cystitis	Neg.	Neg.	Neg.
L. K.	W	M	Neurogenic bladder	Syph.	Doubt.	Neg.
J. R.	W	F	Hunner's ulcer of bladder	Neg.	Neg.	Neg.
L. E.	N	F	Hunner's ulcer of bladder	Neg.	Pos.	6
M. M.	W	M	Hematuria	Neg.	Neg.	Neg.
A. B.	W	M	Hematuria	Neg.	Pos.	120
J. K.	W	M	Congenital vesicorectal fistula	—	—	Neg.

patients with urogenital lesions of questionable origin. Of eleven persons with urethral disease but with no evidence of infection by other venereal agents, four who gave positive Frei reactions and one with a doubtful Frei reaction also showed serum antibodies reacting with Lygranum antigen. In these cases the observed clinical manifestations would appear to be due to lymphogranulomatous infection; instances of abacterial urethritis believed due to this cause are already recorded in the literature.<sup>46</sup> Five additional individuals in the group with urethral lesions (including one with a history of gonorrhea) exhibited complement-fixing antibodies although the Frei reaction was negative. Since it is known that some cases of urethritis may be due to the virus of inclusion conjunctivitis<sup>47</sup> and since the sera of two persons with ocular diseases due to this agent were previously noted to give fixation with Lygranum antigen,<sup>12</sup> it cannot be stated which of these two related viruses may have been responsible for the urethritides in the group with negative Frei reactions.

In twenty-three patients with bladder disturbances, on the other hand, only five showed complement-fixing antibodies versus Lygranum, and four of these also gave positive Frei reactions indicating that they had been infected with lymphogranuloma virus. A more detailed account of the clinical findings in these five patients has been presented by Marshall and Endicott.<sup>48</sup>

#### COMMENT

It seems desirable to summarize here our present information regarding the immunologic features of human lymphogranulomatous infection since this knowledge is prerequisite to the proper interpretation of the data presented in the preceding sections.

Following the introduction of active lymphogranuloma virus in the body, there is usually a relatively prompt appearance of complement-fixing antibodies which may be detected as early as a week after the time of infection<sup>10</sup>; during the subsequent few weeks the titer rises to a maximum, the level of which will vary with the individual. Increase in the serum globulin may also appear prior to the acquisition of cutaneous reactivity<sup>49</sup> which usually, though not invariably, develops after an interval of several weeks of infection.<sup>12, 50, 51</sup> The clinical activity of the individual case would appear to play some, but not necessarily the major, role in determining the intensity of the cutaneous reaction as well as the serologic titer.

In the person who does not receive effective therapy the disease may regress spontaneously or may continue active for long periods of time. Even when the disease becomes clinically healed living virus may continue to reside in the tissues, perhaps dormant but still capable of stimulating the continued production of complement-fixing antibodies and enduring reactivity to the Frei test. Thus patients whose lymphogranulomatous infections date back many years will often give positive cutaneous reactions to Frei or Lygranum antigens<sup>3, 6</sup> as well as complement fixation with the latter<sup>52</sup>; they may still be infectious for their sexual partners and, moreover, active virus has in some instances been recovered experimentally from these cases of long duration.<sup>3</sup> The prolonged stimulation by viral antigen frequently results in the marked synthesis of complement-fix-

ing antibody, this being reflected in a high serum titer and probably also in the extent of the hyperglobulinemia so frequently noted in this disease.<sup>12, 19, 53</sup>

If a patient with early signs of the disease is given vigorous and adequately prolonged treatment with an effective drug such as one of the sulfonamides, the infection may be sterilized. In such case the skin hypersensitivity will wane, and over a period of months one may observe an actual reversal of the Frei reaction<sup>9d, 19, 37, 41</sup>; the level of complement-fixing antibodies also falls gradually, but reactions of low titer may still be demonstrable for many months after the skin reactivity has disappeared.<sup>19</sup> There is evidence that with clinical improvement the elevated serum globulin level may also decline toward the normal.<sup>29, 53d, 53e</sup>

It is well known that there may be considerable variation among individuals in their response to a given antigenic stimulus whether this is encountered in the course of clinical disease or during artificial immunization. Undoubtedly similar variation is shown in the reaction to lymphogranuloma virus, which in most patients appears to constitute a fairly potent antigenic complex. It should not be expected, therefore, that every individual who is infected with the agent will show definite cutaneous reactivity or antibody production, although the vast majority do, in fact, exhibit these responses.<sup>51</sup>

Are these two responses manifestations of a single immunologic mechanism or are they the result of different antigenic stimuli? It is true that some general correlation has been noted between the serum titer and the dimensions of the cutaneous reaction in lymphogranuloma<sup>22</sup>; this may be considered as favoring the first possibility, namely, that the positive Frei reaction depends on the presence of an adequate quantity of complement-fixing antibody in the circulation. The correlation is by no means invariable, however, and there is other evidence which would appear to favor the alternative hypothesis. In the first place, most cutaneous reactions due to the local union of antigen with circulating antibody are of the immediate variety, whereas delayed reactions are seen in the so-called bacterial allergies, wherein passive transfer with antiserum cannot be demonstrated. The Frei reaction is a delayed reaction resembling the reaction to tuberculin or chaneroidal antigen. In the second place, infants born to lymphogranulomatous mothers rarely give positive Frei reactions<sup>55</sup> although passive transfer of complement-fixing antibodies across the placenta occurs regularly.<sup>38, 56</sup> Further, persons infected with other viruses of the psittacosis-lymphogranuloma group may develop potent complement-fixing antibodies which will react with Lygranum, yet fail to give positive cutaneous reactions with this antigen.<sup>12, 57</sup> Finally, we have obtained certain limited evidence (*vide infra*) which suggests that the complement-fixing antibodies may be evoked by inactive virus while the stimulus of living lymphogranuloma virus within the body is a prerequisite for the development of cutaneous reactivity.

Eight normal laboratory workers received, over a period of several months, repeated (up to ten) intradermal and subcutaneous injections of 0.25 to 1.0 ml. of formalinized Lygranum antigen, quantities many times greater than those which would be used in a cutaneous test. Their sera were tested from time to time and in two members of the group no antibodies were found; one person developed a titer of 6; three persons showed a titer of 15; two workers developed a titer of 60. None of the eight showed a positive cutaneous reaction to Lygranum, although lymphogranulomatous patients with a serum titer as low as 6 usually give a positive intradermal reaction, frequently of marked intensity. One of the workers with serum titer of 60 subsequently acquired active infection in the laboratory, following which the skin reaction became strongly positive; the serum titer also showed a further rise. The failure of Lygranum to induce cutaneous hypersensitivity in nonlymphogranulomatous individuals despite repeated injections is in agreement with the behavior of human Frei antigen under similar circumstances.<sup>50, 51, 58</sup>



With the foregoing in mind, we are now in a better position to consider Lygranum complement fixation in the diagnostic study of patients concerning whom the question of lymphogranulomatous infection is raised. We believe the following statements to be justified on the basis of the available data.

Among individuals with authentic lymphogranulomatous disease, irrespective of localization, nearly all will yield positive complement fixation as well as cutaneous tests with Lygranum. Only very rarely will a positive Frei reaction be obtained in the absence of serologic reactivity (see Section 1). By and large the complement fixation test, as we have performed it, appears to be definitely more sensitive and it will therefore detect an appreciable proportion of reactors who fail to yield definitely positive cutaneous tests (see Section 9). The serologic test may become positive before skin hypersensitivity has developed and may persist after the waning of the latter (see Section 8).

There are numerous patients who are asymptomatic but who exhibit definite reactivity to cutaneous and serologic tests with Lygranum. In such persons the demonstration of positive responses in both tests is strong evidence in favor of antecedent infection with the virus of lymphogranuloma, whether or not there are overt signs of Nicolas-Favre disease. Instances of this sort are not infrequently encountered among those who have acquired other venereal disease (see Sections 4 and 5) as well as among marital partners of persons who themselves present evidence of pre-existing lymphogranuloma.

In patients who show complement-fixing antibodies versus Lygranum but who do not react to cutaneous tests, the decision as to diagnostic significance requires consideration of the following possibilities. Some of these serologic reactions may reflect lymphogranulomatous infection in persons with skin anergy<sup>50, 51, 54, 59</sup>; it has been noted in this connection that a temporary reduction in cutaneous reactivity to Frei antigen may sometimes occur during an early syphilitic infection.<sup>54b, 60</sup> In certain cases cutaneous reactivity may not yet have developed or a previously existing reactivity may have faded due to sterilization of the infection while antibodies have persisted as testimony to the former presence of virus (see Section 8). In other instances, the reactions may be due to infection with a related virus such as those of ornithosis, inclusion blennorrhoea, or trachoma (see Section 3). In this group of patients, therefore, the results of a single complement fixation test do not allow a dogmatic conclusion about the nature of the antecedent infection in any individual case; repeated quantitative serologic tests to determine possible alterations in titer with the passage of time, as well as a carefully taken history, may throw additional light on the etiologic background.

It was formerly believed that Nicolas-Favre disease was relatively uncommon and that it nearly always manifested itself in a frank syndrome. During the past fifteen years our knowledge of the nature and extent of the infection has been altered radically. This is due in part to the demonstration of an unexpectedly large number of clinical cases in communities where the prevalence of the disease had not been appreciated previously<sup>16, 61</sup> and to the discovery of a high incidence of positive Frei reactors among those groups in the population

where promiscuity, and poor sexual hygiene frequently eventuate in other venereal infections.<sup>10, 11</sup> The results of complement fixation tests with Lygranum have extended the data previously obtained from cutaneous tests. There is now abundant evidence that the natural history of lymphogranulomatous infection is in some respects not unlike that of tuberculosis or syphilis. All are highly contagious, but in many individuals the infections are so mild that they pass unnoticed unless thorough physical examination and history are obtained; other persons experience subclinical or asymptomatic infections which are only detected by immunologic methods. With the limitations previously noted, the complement fixation test with Lygranum is a valuable epidemiologic tool, since it can serve as a screening device for the selection of those elements in the population which might most profitably be further investigated.

#### SUMMARY

1. In a group of 149 patients who showed a wide variety of overt lesions regarded clinically as lymphogranulomatous and who gave positive cutaneous reactions to lymphogranuloma antigens, the sera of over 98 per cent were found to fix complement in the presence of Lygranum.

2. The antibody titers varied markedly among individuals; differences as great as several hundredfold were observed. It was noted that with the technique employed approximately 10 per cent of the lymphogranulomatous sera reacted, but weakly, giving definitely positive results only in the lowest serum dilution tested (titer 6 by our notation).

3. The limited data available suggested a correlation between the titers of complement-fixing antibodies and the globulin values of lymphogranulomatous sera.

4. Complement-fixing antibodies versus Lygranum were also demonstrated regularly in the sera in several cases of accidental laboratory infection with the virus and in the sera of groups of Frei-positive persons (1) who showed evidence of urogenital localization of lymphogranuloma, or (2) who showed lesions of questionably lymphogranulomatous origin, or (3) who appeared asymptomatic in regard to lymphogranuloma venereum.

5. In healthy individuals (for example, laboratory workers) who were not known to have had contact with viruses of the lymphogranuloma-psittacosis group, the complement fixation test with Lygranum gave negative results. In contrast, the sera of patients who were known to have been infected with other members of this group of related viruses in most instances gave reactions with lymphogranuloma antigens.

6. Reactions were only rarely observed among patients suffering from non-venereal infectious diseases due to agents unrelated to the lymphogranuloma-psittacosis group, irrespective of the site of localization.

7. The sera of patients with neoplasia or cardiovascular or renal disease seldom showed reactions with Lygranum. An appreciable proportion of the sera from a group of patients with evidence of hepatic damage did show such complement fixation; the possible significance of this is discussed.

8. The results of complement fixation tests with Lygranum supported the concept that among those who acquire other genitoinfectious diseases as a consequence of sexual promiscuity there may frequently occur a concomitant infection with the virus of lymphogranuloma venereum.

9. The analysis of the available data indicates that the complement fixation test with Lygranum has certain limitations but may properly be considered useful in diagnostic and epidemiologic studies.

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#### REFERENCES

1. Frei, W.: Eine neue Hautreaktion bei Lymphogranuloma inguinale, *Klin. Wchnschr.* 4: 2148-2149, 1925.
2. Durand, M., Nicolas, J., and Favre, M.: Lymphogranulomatose inguinale subaiguë d'origine génitale probable peut-être vénérienne, *Bull. et mém. Soc. méd. d. hôp. de Paris* 35: 274-288, 1913.
3. Palmer, W. L., Kirsner, J. B., and Rodaniche, E.: Studies on Lymphogranuloma Venereum Infection of the Rectum, *J. A. M. A.* 118: 517-524, 1942.
4. (a) Wassen, E.: Réceptivité de la souris blanche à l'égard du virus lymphogranulomateux (maladie de Nicolas et Favre), *Compt. rend. Soc. de biol.* 114: 493-495, 1933.
- (b) Grace, A. W., and Suskind, F. H.: Successive Transmission of Virus of Lymphogranuloma Inguinale Through White Mice, *Proc. Soc. Exper. Biol. & Med.* 32: 71-72, 1934.
- (c) von Haam, E., and Lichtenstein, L.: Incidence and Clinical Manifestations of Lymphogranuloma Inguinale in New Orleans, *New Orleans M. & S. J.* 88: 92-102, 1935.
- (d) D'Aunoy, R., and von Haam, E.: Diagnostic Value of Frei Reaction in Lymphogranuloma Inguinale, *Am. J. Clin. Path.* 6: 529-545, 1936.
- (e) Nauck, E. G., and Malamos, B.: Ueber Erregerbefunde bei Lymphogranuloma inguinale, *Arch. f. Schiffs- u. Tropen-Hyg.* 41: 537-552, 1937.
5. Jansen, H.: Die Anwendung von Tierantigenen zur Freischen Reaktion (Frei Test), *Arch. f. Schiffs- u. Tropen-Hyg.* 42: 259-263, 1938.
6. Grace, A. W., Rake, G., and Shaffer, M. F.: A New Material (Lygranum) for Performance of the Frei Test for Lymphogranuloma Venereum, *Proc. Soc. Exper. Biol. & Med.* 45: 259-263, 1940.
7. Rake, G., McKee, C. M., and Shaffer, M. F.: Agent of Lymphogranuloma Venereum in Yolk Sac of Developing Chick Embryo, *Proc. Soc. Exper. Biol. & Med.* 43: 332-334, 1940.
8. Rake, G., and Jones, H. P.: Studies on Lymphogranuloma Venereum. I. Development of the Agent in the Yolk Sac of the Chicken Embryo, *J. Exper. Med.* 75: 323-338, 1942.
9. (a) Souza Araujo, H. C. de: Um Novo Antígeno (Lygran), o um Novo medicamento (Sulfathiazole) Para o Diagnóstico e Tratamento do Linfogranuloma Venereo, *An. brasil de dermat. e sif.* 15: 225-227, 1940.
- (b) Sulkin, S. E., Fletcher, P. F., Huber, E. T., and Reh, E. P.: Frei Test for Lymphogranuloma Venereum; Experiences With New Antigen (Lygranum) Prepared From Infected Yolk Sac of Developing Chick Embryo, *J. A. M. A.* 116: 2663-2665, 1941.

- (c) Greenblatt, R. B.: Value of Frei Antigen of Chick Embryo Origin in Diagnosis of Lymphogranuloma Venereum, *J. Oklahoma M. A.* 34: 423-424, 1941.
- (d) Axelrod, S. J.: Diagnosis of Lymphogranuloma Venereum; Evaluation of Virus Antigen (Lygranum) Prepared From Culture on Chick Embryo, *Am. J. Syph., Gonorr. & Ven. Dis.* 26: 474-488, 1942.
- (e) Galvao Peixoto, P.: O Antígeno "Lygranum" e o Novo sulfamidico na Doença de Nicolas-Favre, *An. brasil de dermat. e síf.* 16: 47-57, 1941.
- (f) Combes, P. C., Cañizares, O., and Morris, G.: Frei test; Evaluation of Chick Embryo Antigen (Lygranum), *Arch. Dermat. & Syph.* 46: 261-268, 1942.
- (g) Mortara, F., and Greenblatt, R. B.: Comparison of Antigenicities of Human Pus, Mouse Brain and Chick Embryo Cultures in the Diagnosis of Lymphogranuloma Venereum, *Ven. Dis. Inform.* 24: 14-17, 1943.
- (h) Heisel, E. B., Stroud, G. H., Krause, M., Hubler, W., Cole, H. N., and Cord, R.: The Chick Embryo Antigen (Lygranum) Test for Lymphogranuloma Venereum; a Clinical Investigation, *Arch. Dermat. & Syph.* 48: 379-383, 1943.
10. (a) Robinson, H. M., and Robinson, H. M., Jr.: Comparison of Frei Antigens, *Arch. Dermat. & Syph.* 45: 112-120, 1942.
- (b) Curth, H. C.: Evaluation of Yolk Sac Antigen for Frei Test; Comparison of Tests Performed With Antigens From Yolk Sac Cultures and From Human Pus, *Am. J. Syph., Gonorr. & Ven. Dis.* 27: 47-49, 1943.
11. (a) McKee, C. M., Rake, G., and Shaffer, M. F.: Complement-Fixation Test in Lymphogranuloma Venereum, *Proc. Soc. Exper. Biol. & Med.* 44: 410-413, 1940.
- (b) Shaffer, M. F., Rake, G., and Grace, A. W.: Yolk Sac Antigens in the Diagnosis and Epidemiology of Lymphogranuloma Venereum, *Am. J. Syph., Gonorr. & Ven. Dis.* 26: 271-281, 1942.
12. Rake, G., Shaffer, M. F., and Thygeson, P.: Relationship of Agents of Trachoma and Inclusion Conjunctivitis to Those of Lymphogranuloma-Psittacosis Group, *Proc. Soc. Exper. Biol. & Med.* 49: 545-547, 1942.
13. Rake, G., Eaton, M. D., and Shaffer, M. F.: Similarities and Possible Relationships Among Viruses of Psittacosis, Meningo-Pneumonitis, and Lymphogranuloma Venereum, *Proc. Soc. Exper. Biol. & Med.* 48: 528-531, 1941.
14. (a) Eaton, M. D., and Corey, M.: Complement-Fixation in Human Pneumonitis With Group-Reactive Virus Antigens, *Proc. Soc. Exper. Biol. & Med.* 51: 165-168, 1942.
- (b) Smadel, J. E., Wertman, K., and Reagan, R. L.: Yolk Sac Complement Fixation Antigen for Use in Psittacosis-Lymphogranuloma Venereum Group of Diseases, *Proc. Soc. Exper. Biol. & Med.* 54: 70-74, 1943.
- (c) Smadel, J. E.: Atypical Pneumonia and Psittacosis, *J. Clin. Investigation* 22: 57-65, 1943.
15. (a) Eaton, M. D., Martin, W. P., and Beck, M. D.: The Antigenic Relationship of the Viruses of Meningopneumonitis and Lymphogranuloma Venereum, *J. Exper. Med.* 75: 21-33, 1942.
- (b) Beck, M. D., Eaton, M. D., and O'Donnell, R.: Further Laboratory Studies on the Classification of Psittacosis-like Agents, *J. Exper. Med.* 79: 65-77, 1944.
16. Nigg, C.: Action of Urea and Ether on Agent and Complement-Fixing Antigen of Lymphogranuloma Venereum, *Proc. Soc. Exper. Biol. & Med.* 49: 132-135, 1942.
17. (a) Nigg, C., and Bowser, B. M.: Enhancement of Phenol of Serological Reactivity of Lymphogranuloma Venereum Antigens, *Proc. Soc. Exper. Biol. & Med.* 53: 192-194, 1943.
- (b) Nigg, C., Hilleman, M. R., and Bowser, B.: Studies on Lymphogranuloma Venereum Antigens. I. Enhancement by Phenol or Boiling, *J. Immunol.* 53: 259-268, 1946.
- (c) Bowser, B., and Nigg, C.: Studies on Lymphogranuloma Venereum Antigens. II. Serological Studies With Boiled Phenolized Antigens, *J. Immunol.* 53: 269-275, 1946.
18. Shaffer, M. F., Rake, G., and McKee, C. M.: Agent of Lymphogranuloma Venereum in the Lungs of Mice, *Proc. Soc. Exper. Biol. & Med.* 44: 408-410, 1940.
19. (a) Harrop, G. A., Rake, G. W., and Shaffer, M. F.: A Group of Laboratory Infections Ascribed to Lymphogranuloma Venereum, *Tr. Am. Clin. & Climatol. A.* 56: 154-159, 1941.
- (b) Harrop, G. A., Rake, G. W., and Shaffer, M. F.: New Clinical Conceptions of Lymphogranuloma Venereum, *Tr. A. Am. Physicians* 56: 101-105, 1941.
20. Rake, G., Shaffer, M. F., Jones, H. P., and McKee, C. M.: Soluble Antigen in Lymphogranuloma Venereum, *Proc. Soc. Exper. Biol. & Med.* 46: 300-303, 1941.
21. Casals, J., and Palacios, R.: The Complement Fixation Test in the Diagnosis of Virus Infections of the Central Nervous System, *J. Exper. Med.* 74: 409-426, 1941.
22. Grace, A. W., and Rake, G.: The Complement Fixation Test for Lymphogranuloma Venereum; Results Obtained With Its Use, *Arch. Dermat. & Syph.* 48: 619-625, 1943.
23. Dulany, A. D., and Packer, H.: Observations Upon the Specificity of the Complement Fixation Test for Lymphogranuloma Venereum, *J. Immunol.* 55: 53-60, 1947.

24. Levine, S., Holder, E. C., and Bullowa, J. G. M.: Complement Fixation for Lymphogranuloma Venereum and for Psittacosis With Frei Reactions Among Pneumonia Patients, *J. Immunol.* 46: 183-188, 1943.
25. Blair, J. E.: The Complement-Fixation Reaction With the Antigen of Lymphogranuloma Venereum (Lygrauum), *J. Immunol.* 49: 63-70, 1944.
26. Knott, L. W., Bernstein, L. H. T., Eagle, H., Billings, T. E., Zobel, R. L., and Clark, E. G.: The Differential Diagnosis of Lymphogranuloma Venereum and Chancroid by Laboratory and Skin Tests, *Am. J. Syph., Gonorr. & Ven. Dis.* 27: 657-665, 1943.
27. Howard, M. E., Benham, O. R., and Borman, E. K.: The Complement Fixation Test in Lymphogranuloma Venereum, *Federation Proc.* 4: 165-166, 1945.
28. Florman, A. L.: The Use of a Commercially Available Complement Fixing Antigen for the Diagnosis of Elementary Body Types of Viral Infection, *J. Immunol.* 51: 29-37, 1945.
29. Schamberg, I. L.: The Course of the Plasma Protein Changes in Early Lymphopathia Venereum Under Treatment With Sulfanilamide, *Am. J. M. Sc.* 201: 67-81, 1941.
30. Francis, T., Jr., and Magill, T. P.: An Unidentified Virus Producing Acute Meningitis and Pneumonitis in Experimental Animals, *J. Exper. Med.* 68: 147-160, 1938.
31. (a) Gönner, R.: Ueber ein Neues, dem Erreger des Lymphogranuloma inguinale ähnliches Mausevirus, *Klin. Wchenschr.* 20: 76-78, 1941.  
(b) Nigg, C.: An Unidentified Virus Which Produces Pneumonia and Systemic Infection in Mice, *Science* 95: 49-50, 1942.  
(c) Nigg, C., and Eaton, M. D.: Isolation From Normal Mice of a Pneumotropic Virus Which Forms Elementary Bodies, *J. Exper. Med.* 79: 497-510, 1944.
32. (a) Meyer, K. F.: Ecology of Psittacosis and Ornithosis, *Medicine* 21: 175-206, 1942.  
(b) Eddie, B., and Francis T., Jr.: Occurrence of Psittacosis-like Infection in Domestic and Game Birds of Michigan, *Proc. Soc. Exper. Biol. & Med.* 50: 291-295, 1942.
33. (a) Eaton, M. D., Beck, M.D., and Pearson, H. E.: A Virus From Cases of Atypical Pneumonia; Relation to the Viruses of Meningopneumonitis and Psittacosis, *J. Exper. Med.* 73: 641-654, 1941.  
(b) Larson, C. L., and Olson, B. J.: An Epidemic of a Severe Pneumonitis in the Bayou Region of Louisiana. VI. A Comparative Study of the Viruses of Lymphogranuloma Venereum, Psittacosis and Louisiana Pneumonitis, *Pub. Health Rep.* 61: 69-78, 1946.
34. Dingle, J. H., Abernathy, T. J., Badger, G. F., Buddingh, G. J., Feller, A. E., Langmuir, A. D., Rueggsegger, J. M., and Woods, W. B., Jr.: Primary Atypical Pneumonia, Etiology Unknown, *Am. J. Hyg.* 39: 197-336, 1944.
35. Thomas, L., Curnen, E. C., Mirick, G. S., Ziegler, J. E., Jr., Horsfall, F. L., Jr.: Complement Fixation With Dissimilar Antigens in Primary Atypical Pneumonia, *Proc. Soc. Exper. Biol. & Med.* 52: 121-125, 1943.
36. Grace, A. W., Shaffer, M. F., and Rake, G.: Further Evidence Concerning the Specificity of the Lymphogranuloma Venereum Complement Fixation Test in Syphilis, *Am. J. Syph., Gonorr. & Ven. Dis.* 27: 44-46, 1943.
37. Koteen, H.: Lymphogranuloma Venereum, *Medicine* 24: 1-69, 1945.
38. Beeson, P. B., and Miller, E. S.: Epidemiological Study of Lymphogranuloma Venereum, Employing the Complement Fixation Test, *Am. J. Pub. Health* 34: 1076-1082, 1944.
39. Rake, G., and Jones, H. P.: Studies on Lymphogranuloma Venereum. II. The Association of Specific Toxins With Agents of the Lymphogranuloma-Psittacosis Group, *J. Exper. Med.* 79: 463-486, 1944.
40. Rake, G., and Jones, H. P.: A Toxic Factor Associated With the Agent of Lymphogranuloma Venereum, *Proc. Soc. Exper. Biol. & Med.* 53: 86-88, 1943.
41. Curth, W., and Curth, H.: Hepatosplenomegaly Probably Due to Lymphogranuloma Venereum, *Arch. Dermat. & Syph.* 45: 1193-1194, 1942.
42. Cardon, L., and Atlas, D. H.: Incidence and Causes of Hyperproteinemia; a Study of 1,390 Cases, *Arch. Int. Med.* 71: 377-390, 1943.
43. Oliphant, J. W., Powell, W. F., and Perrin, T. L.: Sulfadiazine in Lymphogranuloma Venereum Ophthalmitis, *J. A. M. A.* 118: 973-974, 1942.
44. Stein, R. O.: Observations on Thirty-five Cases of Venereal Lymphogranuloma Treated With Sulfanilamide, *Am. J. Syph., Gonorr. & Ven. Dis.* 24: 454-467, 1940.
45. Findlay, G. M.: Climatic Bubo or Lymphogranuloma Inguinale—Experimental Investigation, *Tr. Roy. Soc. Trop. Med. & Hyg.* 31: 587-600, 1938.
46. (a) Gray, L. A.: Lymphopathia Venereum—"Lymphogranuloma Inguinale"—of the Female Urethra, *Surg., Gynec. & Obst.* 62: 745-751, 1936.  
(b) Birrozera, F., and Midana, A.: Sur la localisation uretrale de la maladie de Nicolas et Favre, *Ann. de dermat. et syph.* 9: 849-856, 1938.  
(c) May, J.: Poradenolinfitis, Montevideo, 1940 and 1943, *El Siglo Ilustrado*.  
(d) Costello, M. J., and Cohen, J. A.: Lymphogranuloma Venereum; Observations in 187 Cases at Bellevue Hospital, *Arch. Dermat. & Syph.* 44: 391-397, 1941.

- (e) Coutts, W. E.: Genito-Urinary Lesions in Lymphogranuloma Venereum, *J. Urol.* 49: 595-598, 1943.
47. Thygeson, P., and Mengert, W. F.: The Virus of Inclusion Conjunctivitis—Further Observations, *Arch. Ophth.* 15: 377-410, 1936.
48. Marshall, V. F., and Endicott, E.: A Clinical Study of Obscure Bladder Disease Using Frei Tests, *J. Urol.* 50: 76-81, 1913.
49. Kampmeier, R. H., Smith, D. W., and Larsen, R. M.: Blood Studies in Lymphogranuloma Venereum, With Special Reference to Serum Proteins, *Am. J. M. Sc.* 198: 516-528, 1939.
50. Kornblith, B. A.: Lymphogranuloma Venereum, *Arch. Dermat. & Syph.* 44: 729-731, 1941.
51. Connor, W. H., Levin, E. A., and Ecker, E. E.: Observations on the Frei Test, *J. Infect. Dis.* 60: 62-63, 1937.
52. Rake, G., Shaffer, M. F., Grace, A. W., McKee, C. M., and Jones, H. P.: New Aid in the Diagnosis of Lymphogranuloma Venereum, *Am. J. Syph., Gonorr. & Ven. Dis.* 25: 687-698, 1911.
53. (a) Williams, R. D., and Gutman, A. B.: Hyperproteinemia With Reversal of the Albumin:Globulin Ratio in Lymphogranuloma Inguinale, *Proc. Soc. Exper. Biol. & Med.* 34: 91-94, 1936.
- (b) Kendall, F. G.: The Use of Immunochemical Methods for the Identification and Determination of Human Serum Proteins, Cold Spring Harbor Symposia on Quant. Biol. 6: 376-381, 1938.
- (c) Rosen, I., Rosenfeld, H., Bloom, D., and Krasnow, F.: Lymphogranuloma Venereum—Serum Lipids and Proteins, *Arch. Dermat. & Syph.* 39: 211-216, 1939.
- (d) Howard, M. E., Eisenman, A. J., and Strauss, M. J.: Alterations in the Serum Proteins in Lymphopathia Venerea, *Am. J. Syph., Gonorr. & Ven. Dis.* 23: 83-96, 1939.
- (e) Jones, C. A., and Rome, H. P.: Serum Proteins, Takata-Ara Reaction and Liver Function Tests in Lymphogranuloma Venereum, *Am. J. Clin. Path.* 9: 121-136, 1939.
- (f) Beeson, P., and Miller, E. S.: Relationship of Lymphogranuloma Venereum Infection to the Incidence of Hyperglobulinemia, *Am. J. M. Sc.* 207: 613-616, 1944.
54. (a) Frei, W.: On the Skin Test in Lymphogranuloma Inguinale. A Brief Review and a Discussion of Some Possible Causes of Error, *J. Invest. Dermat.* 1: 367-377, 1938.
- (b) Frei, W.: Combatting Lymphogranuloma Venereum, *Arch. Dermat. & Syph.* 47: 830-843, 1943.
55. Wilson, C. L., and Hesseltine, H. C.: Effect of Lymphogranuloma Venereum on Pregnancy, Labor and the Fetus, *Am. J. Obst. & Gynec.* 43: 459-467, 1942.
56. Levine, S., Bullowa, J. G. M., and Scheinblum, I. E.: Antepartum Transmission of Lymphogranuloma Venereum Antibodies and Their Duration in the Infant, *J. Immunol.* 47: 439-442, 1943.
57. Davis, W. A., and Dingle, J. H.: Personal communications.
58. Wassen, E.: Studies of Lymphogranuloma Inguinale From Etiological and Immunological Points of View, *Acta path. et microbiol. Scandinav.* suppl. 23, pp. 1-181, 1935.
59. Sabin, A. B., and Aring, C. D.: Meningoencephalitis in Man Caused by the Virus of Lymphogranuloma, *J. A. M. A.* 120: 1376-1381, 1942.
60. (a) Cole, H. N.: Lymphogranuloma Inguinale, the Fourth Venereal Disease, *J. A. M. A.* 101: 1069-1076, 1933.
- (b) Bacon, H. E.: The Specificity of the Frei Test in Lymphopathia Venerea, *Am. J. Digest. Dis. & Nutrition* 2: 570-571, 1935.
61. (a) DeWolf, H. F., and van Cleave, J. V.: Lymphogranuloma Inguinale, *J. A. M. A.* 99: 1065-1071, 1932.
- (b) Gray, S. H., Hunt, G. A., Wheeler, P., and Blache, J. O.: Lymphogranuloma Inguinale: Its Incidence in St. Louis, *J. A. M. A.* 106: 919-920, 1936.
- (c) Haim, A., and Mathewson, C.: Incidence of Lymphogranuloma Inguinale in San Francisco, *J. A. M. A.* 108: 961-965, 1937.

# COAGULATION TIME OF BLOOD HEPARINIZED IN VITRO: CORRELATION OF RESULTS WITH THOSE OF THE HEPARIN TOLERANCE TEST

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RECENTLY we reported on a heparin tolerance test<sup>1</sup> and gave results obtained in a study of normal subjects and patients suffering from various types of intravascular thrombosis. This paper is presented to show the results obtained with a test of coagulation time of whole blood heparinized in vitro and to compare these results with those obtained with the more complex heparin tolerance test.

The idea of determining coagulation time after adding heparin to blood in vitro rather than in vivo was suggested by papers published by Waugh and Ruddick.<sup>2, 3</sup> These workers used a series of test tubes containing progressively increasing amounts of heparin, each of which contained 1 ml. of venous blood. After the coagulation time of the blood in each of the tubes was determined, a curve was charted and from this they derived their conclusions. We wished to establish a simpler procedure.

If the blood volume of an average normal adult were 5 liters, 25 mg. of heparin given intravenously for a heparin tolerance test would produce a concentration of 0.005 mg. of heparin per cubic centimeter of blood ten minutes after injection, at which time the blood and heparin should be mixed completely. Arbitrarily then, we decided to determine the coagulation times of 1 ml. of blood drawn from normal persons and patients suffering from intravascular thrombosis when the blood was added immediately after withdrawal to 0.005 mg. of heparin. Obviously, after an intravenous injection of 25 mg. of heparin the concentration of heparin is not always 0.005 mg. per milliliter of blood, since the blood volume of adult individuals varies. Hence, a slight discrepancy might be expected in comparing the coagulation times of blood obtained in the heparin tolerance test and in the test of heparinized blood in vitro.

The coagulation test of blood heparinized in vitro was performed as follows. Three 10 by 75 mm. glass tubes were used for each test. One tube was left empty. In the second tube, 0.5 ml. of a 0.9 per cent solution of sodium chloride without heparin was placed. In the third tube, 0.005 mg. of heparin dissolved in 0.5 ml. of 0.9 per cent solution of sodium chloride was placed. Blood was withdrawn from the antecubital vein and 1 ml. was measured carefully and placed in each of the three tubes. The second and third tubes were vigorously inverted three times as soon as the blood was added in order to mix the blood

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TABLE I. COAGULATION TIME IN MINUTES OF TWENTY-SIX NORMAL SUBJECTS

SUBJECT	AGE	SEX	WEIGHT	1 ML. OF BLOOD	1 ML. OF BLOOD AND 0.5 ML. 0.9 PER CENT SODIUM CHLORIDE	1 ML. OF BLOOD, 0.5 ML. 0.9 PER CENT SODIUM CHLORIDE, AND 0.005 MG. HEPARIN	1 ML. OF BLOOD 10 MIN. AFTER INTRAVENOUS INJECTION OF 25 MG. HEPARIN
1	36	F	128	5.5	6.0	61	61
2	38	F	127	5.5	5.5	58	53
3	20	F	120	6.0	6.0	51	16
4	17	F	153	1.5	5.0	55	47
5	19	F	129	6.5	6.5	93	82
6	25	F	126	1.0	4.0	37.5	54
7	27	F	112	5.0	5.5	45	54
8	20	F	144	6.5	6.5	63	51
9	21	F	125	8.0	9.0	65	63
10	34	F	138	9.0	8.5	77	65
11	46	F	162	7.5	8.0	75	66
12	38	M	154	6.5	5.5	84	72
13	48	F	143	8.0	7.5	62	66
14	25	F	122	7.5	7.0	58	53
15	22	F	110	7.0	7.5	61	57
16	33	F	128	9.5	9.0	82	68
17	28	F	117	6.0	6.0	47	56
18	24	F	124	6.0	7.5	54	46
19	28	F	116	9.0	8.5	44	66
20	58	F	134	8.0	7.5	59	81
21	24	F	138	12.0	13.0	55	52
22	24	F	118	7.0	8.0	52	59
23	45	F	148	9.0	9.0	72	63
24	60	M	182	6.0	6.5	16	53
25	29	F	132	6.0	6.0	50	57
26	41	M		11.0	10.0	76	64

TABLE II. COAGULATION TIME IN MINUTES IN NINETEEN CASES OF THROMBOSIS

SUBJECT	AGE	SEX	WEIGHT	1 ML. OF BLOOD	1 ML. OF BLOOD, 0.5 ML. 0.9 PER CENT SODIUM CHLORIDE, AND 0.005 MG. HEPARIN	1 ML. OF BLOOD 10 MIN. AFTER INTRAVENOUS INJECTION OF 25 MG. HEPARIN
27	38	M	149	6.0	24	18
28	51	M	184	6.5	21	22
29	56	M	-	8.0	36	44
30	65	M	128	9.0	98	74
31	57	M	180	7.5	14	11
32	57	M	135	5.0	8	10
33	39	M	129	5.5	58	52
34	53	M	146	7.0	60	68
35	28	M	153	8.0	64	59
36	50	M	-	8.5	33	37
37	40	M	190	6.5	51	56
38	44	M	163	6.0	51	46
39	34	M	118	7.0	75	69
40	38	M	165	6.5	10	11.5
41	33	M	140	7.0	10	9
42	38	M	151	6.0	16	10
43	33	M	140	6.0	32	43
44	38	M	151	5.5	31	34
45	33	M	160	8.0	35	40



TABLE III. REPEATED COAGULATION TIMES IN MINUTES ON DIFFERENT DATES IN THREE NORMAL SUBJECTS

SOLUTION TESTED	SUBJECT 1 AGE, 38; SEX, F; WEIGHT, 122					SUBJECT 2 AGE, 18; SEX, F; WEIGHT, 131				SUBJECT 3 AGE, 26; SEX, F; WEIGHT, 117			
	FEB. 22	MAR. 5	MAR. 9	APR. 1	APR. 11	FEB. 22	MAR. 5	APR. 1	APR. 11	FEB. 22	MAR. 5	MAR. 9	APR. 11
1 ml. of blood	4.5	6.5	7.5	5	5.5	5.5	6	5	5.5	7	7	7	7
1 ml. of blood and 0.5 ml. 0.9 per cent sodium chloride	5	6.5	8.0	5	6	5.5	6	5.5	5.5	7	8	7.5	7.5
1 ml. of blood, 0.5 ml. 0.9 per cent sodium chloride, and 0.005 mg. heparin	50	54	51	56	48	56	57	54	52	59	51	59	46
1 ml. of blood 10 min. after intravenous injection of 25 mg. heparin	56	63	54	60	64	51	59	48	64	48	44	52	57

with the solutions. The timing was begun as soon as the blood was drawn from the vein into the syringe. All tubes were inverted at thirty-second intervals until clotting occurred as in the heparin tolerance test. All tests were carried out at hospital room temperatures. Inasmuch as some question arose concerning loss of potency of the dilute solution of heparin used in the test, a fresh solution of the heparin (10 mg. in 1,000 ml. of 0.9 per cent solution of sodium chloride) was made every seven days or oftener as needed.

The coagulation time of blood heparinized (0.005 mg. per milliliter) in vitro was determined, and the heparin tolerance test was carried out successively on the same day in forty-five persons. The results of the two tests are compared in Tables I and II.

The coagulation time of 1 ml. of blood did not differ significantly from that of 1 ml. of blood mixed with 0.5 ml. of 0.9 per cent solution of sodium chloride (Tables I and III).

In this study the coagulation times of blood heparinized in vitro and in vivo were essentially similar for each of the persons who were tested. Relatively slight differences may be explained by variations in blood volumes from 5,000 ml. in different individuals and also are probably within the limits of the experimental error inherent in either method.

The results of the heparin tolerance test can be closely duplicated by testing the coagulation time of blood added to heparin in vitro immediately after withdrawal if the concentration of heparin is approximately the same. The coagulation time of heparinized blood in vitro is a simpler test.

## REFERENCES

1. Hagedorn, A. B., and Barker, N. W.: Response of Persons With and Without Intravascular Thrombosis to a Heparin Tolerance Test, *Am. Heart J.* In press.
2. Waugh, T. R., and Ruddick, D. W.: Test for Increased Coagulability of Blood, *Canad. M. A. J.* 50: 547-549, 1944.
3. Waugh, T. R., and Ruddick, D. W.: Studies on Increased Coagulability of the Blood, *Canad. M. A. J.* 51: 11-17, 1944.

# A NEW METHOD FOR THE INDIRECT MEASUREMENT OF BLOOD PRESSURE IN THE RAT

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VARIOUS methods for the indirect measurement of blood pressure of the rat have been reported in the literature<sup>1,2</sup> and summarized in *The Rat in Laboratory Investigation*.† It has become common practice to determine the blood pressure of the rat by the method of Williams, Harrison, and Grollman.<sup>2</sup> The divergent and inconsistent results obtained by comparison of both direct and indirect blood pressure measurements led Schroeder<sup>4</sup> to doubt the validity of the various indirect methods of measurement of blood pressure in the rat. Shuler, Kupperman, and Hamilton<sup>5</sup> attributed these discrepancies to the size of the sphygmometer cuff applied to the base of the tail and to the sensitivity of the plethysmograph. By changing the membrane of the plethysmograph to a much finer membrane, and by making the size of the oncometer tube attached to the plethysmograph much smaller ( $\frac{1}{2}$  mm.), they were able to increase the sensitivity of the instrument. However, these workers did not give any blood pressure values for unheated, unanesthetized normal animals obtained with these changes, although many values were reported for the anesthetized normal animal. Proskauer, Neumann, and Graef<sup>6</sup> studied independently the problem of these discrepancies. Using the apparatus of Williams and co-workers,<sup>2</sup> modified so as to be in agreement with the changes recommended by Shuler and associates,<sup>5</sup> they came to the following conclusions. "The systolic pressure of trained, normal unanaesthetized rats, as measured by a modification of the indirect method in which there was no change in the rectal or cutaneous temperature, was 65 to 95 mm. Hg at room temp. 24-28° C." These blood pressure values were still inconsistent with those of direct puncture, although Shuler and co-workers got higher values by the indirect method which agreed with the direct method when the rats were anesthetized.

Since we had been aware since 1943 of the discrepancy between the direct and indirect methods of blood pressure measurement, we also independently sought methods by which these disagreements could be explained. On the assumption that the tail method of Williams and co-workers was fundamentally sound, we attributed our inability to use the method to the relative inability of the described apparatus to measure the small volume changes taking place in the tail before and after constriction of the sphygmometer cuff. To overcome this obstacle, we resorted to a very sensitive photoelectric system by which the change in volume of the tail was measured by the change in the size of a slit formed between a metal knife-edge adjusted to form the slit by nearly touching the upper surface of the tail. The distance of the upper surface of the tail from

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†Griffith, John Q. Jr., and Farriss, Edmond J. (editors): *The Rat in Laboratory Investigation*, Philadelphia, 1942, J. B. Lippincott Co.

the metal knife-edge depends on the total volume of the tail which changed on lowering or raising the pressure in a sphygmometer cuff above or below the systolic pressure. Even with this extremely sensitive system the necessity of heating normal unanesthetized animals in order to obtain volume changes in the tail was not completely overcome. The results obtained<sup>7</sup> were very much in agreement with those of Proskauer and co-workers. During the course of our experiments, it was observed that:

1. Even with our more sensitive method of recording volume changes in the tail, we were generally unable to obtain volume changes without heating the animal.

2. As the rats were warmed, readings could gradually be obtained, the volume change being greater as more heat was applied. However, these volume changes did not occur at the same sphygmometer pressure-cuff pressure but at higher indicated pressures. There seemed to be a proportionality between temperature and observed blood pressure reading.

3. When the rats were heated excessively the blood pressure reading fell suddenly to very low levels.

4. In attempting to standardize the rate of heating, different rats responded differently to the same heating conditions, and the same rat responded differently on different days to given heating conditions.

5. There were no optimal conditions under which comparable blood pressures were obtained in the same rat or in different normal rats.

6. Rats which gave no indirect readings at normal room temperature, when nembutalized, gave indirect blood pressure readings at normal room temperature and higher readings when heated under anesthesia.

For these reasons, it was obvious that a more dependable method of indirect blood pressure measurement would have to be developed if the rat were to be used for chronic blood pressure studies. This method would have to fill the following prerequisites:

- (1) The indirect method of determining the blood pressure must be on some appendage other than the tail, in order to obviate the "reflex vasoconstriction" of the arteries of the tail which was influenced by heating or anesthetizing the animal.

- (2) The normal, unheated, unanesthetized animal must be used in order to eliminate all contributing factors which would influence blood pressure.

- (3) In taking the blood pressure, control of all physical factors (light, handling, type of restraint, etc.) which would affect blood pressure must be possible.

After approximately one and one-half years of further study an instrument and a technique was developed which met the stated requirements. It depends on (1) use of the foot (Griffith's technique),<sup>1</sup> and (2) measurement of volume change (Byrom and Wilson,<sup>2</sup> and Williams and associates<sup>3</sup>) of the foot (Griffith) by means of the change in volume and intensity of light as measured by a photocell-microammeter arrangement.

The instrument and technique which have eventually given us the mentioned prerequisites are described below.

*The Rat Holder.*—The restraining cage has been developed with a view to comfort and darkness (for quietness); it is well ventilated and so constructed as to leave the two hind legs suspended freely at the groin. The "head" part of the cage is of metal with a large overlapping cover. This cover is not airtight and permits free entrance of air but not light. At approximately the chest region of the rat a leather "body holder" is fixed to the metal head case with a clamp. This leather is split along the "back." At the "tail end" of the cage two holes are made through the leather to permit the legs to hang. When the rat is in the cage the split on the "back" of the cage is held closed by means of clips. When the rat has been in the cage for a minute or so, it usually relaxes and remains quite still (see Fig. 1).

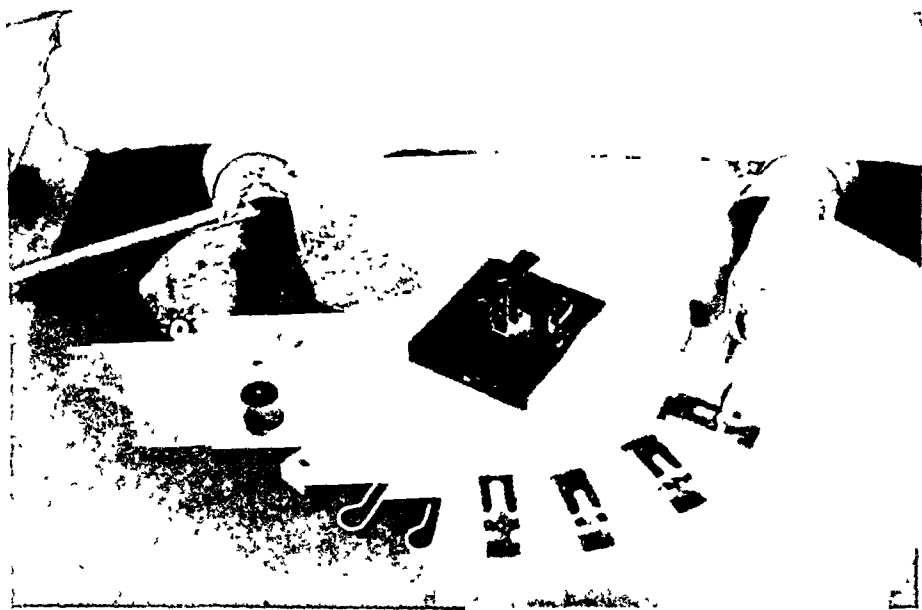


Fig. 1.—Photograph of rat holder and various types of rat foot holders.

*The Sphygmometer Cuff.*—The type of sphygmometer cuff necessary required standardization in making them. A study has been made of different cuffs varying in cross-sectional thickness of the wall and in cross-sectional diameter of the tube itself.

The cuffs are made as follows:

Natural Latex\* is well emulsified and filtered through cheesecloth into a graduate. It is allowed to stand for one hour, after which time any minute air bubbles in the top of the emulsion are brushed off with scraps of filter paper.

Glass rods of the desired diameter, approximately ten to twelve inches long, are cleaned well, rinsed with distilled water, and then rinsed with 3 per cent ammonia water. They are allowed to air-dry and then are dipped into the latex. The rods are carefully (without touching the sides of the graduate) with-

\*Obtained from General Latex and Chemical Co., Cambridge, Mass.

drawn at a constant rate of speed. (A geared electric motor with a spindle is very desirable.) The rate of withdrawal of the rod determines the wall uniformity and thickness of the rubber tube. Usually four inches a minute has been the satisfactory speed. A single dipping will give a thin rubber tube which does not stand excessive use. After permitting the rod to air-dry for 1.5 to 2 minutes, the rod is again immersed and withdrawn at the same speed. It is allowed to air-dry as before. Any minute air bubbles may be removed from the rod by blowing gently on the rod or by carefully touching the air bubbles with the torn edge of a piece of filter paper. The rod may be dipped as frequently as desired for building up the wall thickness.

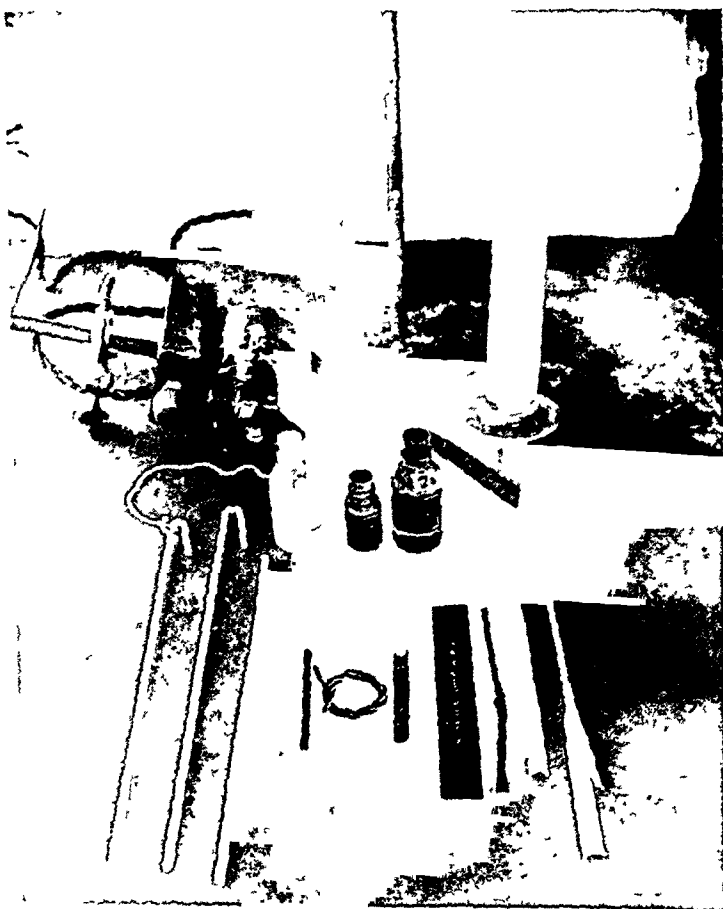


Fig. 2—Preparation of sphygmometer cuffs.

The rod is then allowed to air-dry for seventy-two to ninety-six hours, or the process may be speeded up by heating in an oven at 99 to 100° C. for four to six hours. When dry, the rubber tubing is removed from the rod by lubricating thoroughly with a glycerine (3 Gm.)-talc (1 Gm.) mixture both externally and internally as it is freed from the rod. When completely free of the rod, the rubber tube is washed with water, dried, and lubricated with talc. Sections of desired length may be cut as needed.

The size cuff most practical and efficient has been found to be a 2-dip, five-inch per minute 5 mm. diameter tube. A length of approximately 7 cm. is cut off and sealed at one end with rubber cement. The other end is joined and cemented to 8 to 12 cm. of 5 mm. catheter tube. A 9 cm. piece of one-fourth or five-sixteenth inch ribbon is also cemented to the catheter by winding with a 5 mm. wide strip of latex cut from a sheet (see Fig. 2).

Cuffs of varying outside diameters and wall thicknesses have been prepared, and corresponding variations in the indicated blood pressure were determined. When cuffs less than 4 mm. diameter (when flattened out) are made by a 2- or more, dip procedure, the wall thickness begins to play a role and excessively high recorded pressures ensue since the resistance of the rubber must be overcome in order to compress off the circulation into the foot. An initial standardization of cuffs, making a comparison with a direct puncture, would be advisable for one who has not had previous experience to be sure that the cuffs are of the proper thickness.

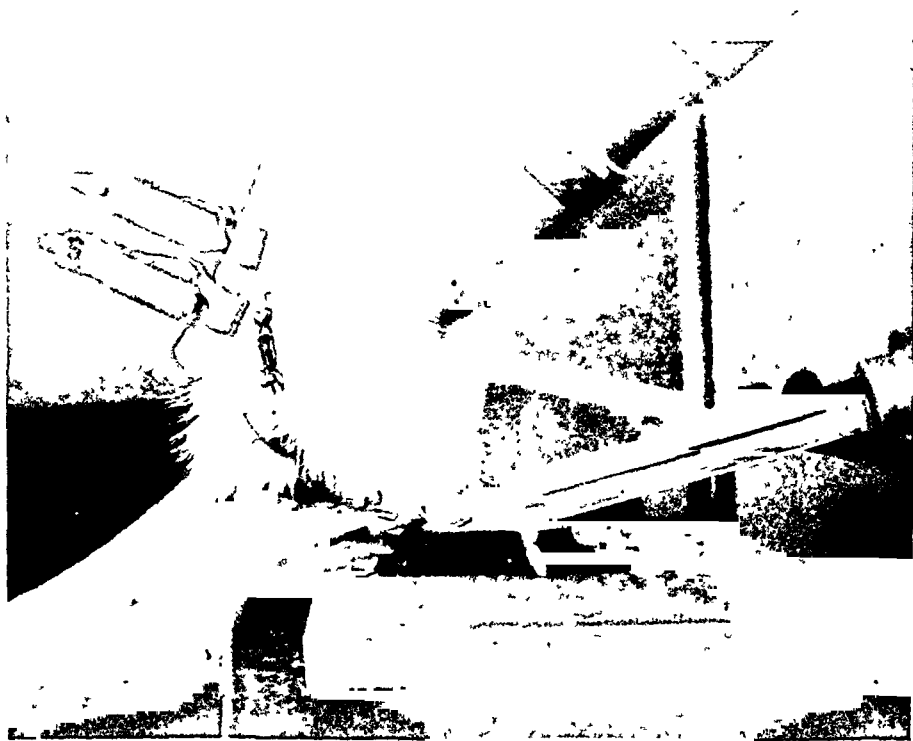


Fig. 3.—Illustration of positions of foot, foot holder, cuff, light, and position of rat for taking the blood pressure.

*The Photocell-Microammeter Volume Change Indicator.*—As shown in Fig. 3, the only light striking the photocell is that which passes through the foot from the light source. This value of light will remain constant as long as the foot is still and allowed to function normally. But when the flow of blood leaving the foot is restricted, the foot will begin to swell, causing a decrease in the amount of light striking the photocell. The circuit for the indicator is shown in Fig. 4. High impedance pentode tube acts as a load impedance for the high

impedance vacuum type photocell. The characteristic curves (Fig. 5) show that the potential of the common connection between the phototube and the pentode is determined by the intersection of their characteristics. It is also evident that a very small change of light on the photocell will result in a correspondingly large voltage change. This output voltage is applied to the grid

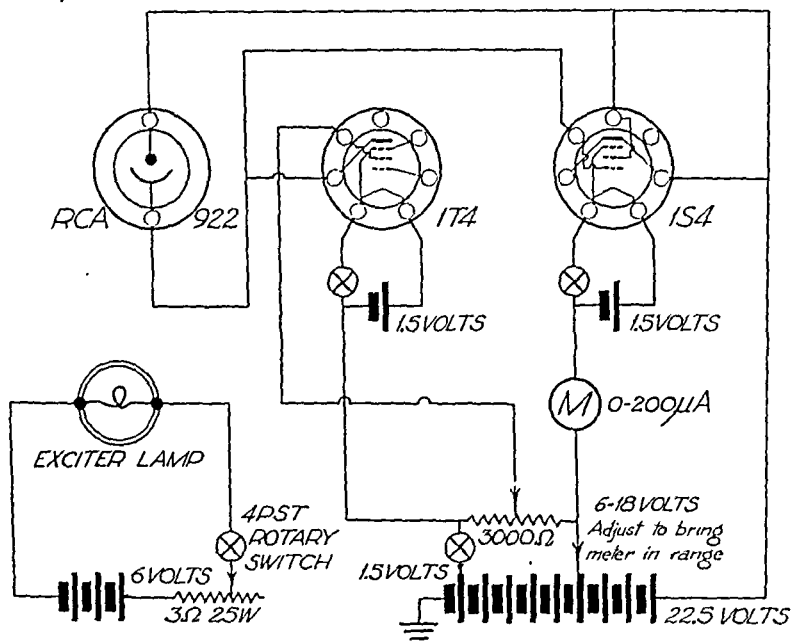


Fig. 4.—Circuit of the photocell microammeter volume change indicator.

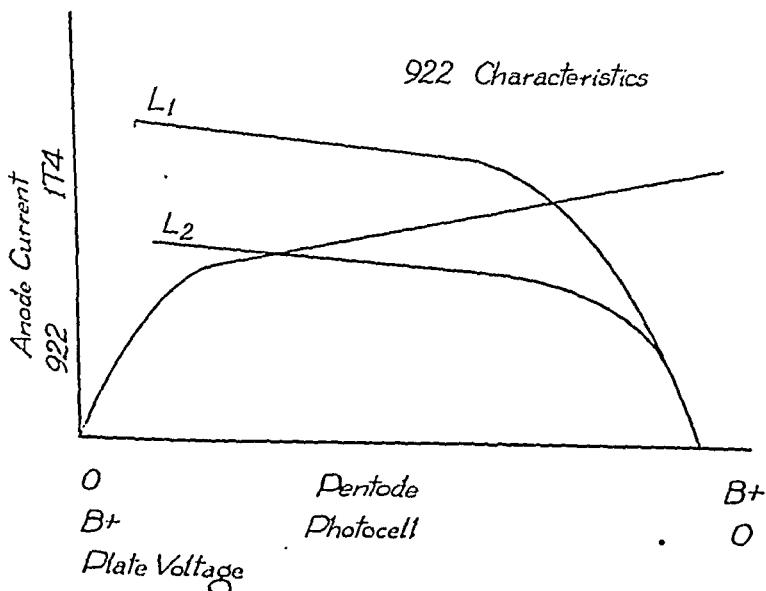


Fig. 5.

of the 1S4 output tube, the plate current of which is indicated on the 0 to 200 microammeter.<sup>8</sup>

*The Foot Holder.*—It must be pointed out that the footholder was designed not to hold the foot in position forcibly, but only to restrain if the rat attempts to withdraw the foot, so that the foot does not leave its correct position. This foot holder is not absolutely necessary but has been an aid in training the rats, and once trained they are conditioned to its feel. A hole directly under the place where the "palm" of the rat's foot rests is also made. This leads directly over the photocell. (See Figs. 1 and 3.)

*Sensitivity.*—By sensitivity is meant the responsiveness of the instrument to a given change in amount of light hitting the photocell. This is taken care of by the circuit itself.

*Accuracy.*—By accuracy is meant the amount of lag between the first change in volume in the foot caused by lowering the pressure of the sphygmometer cuff and the recording of the change in volume on the galvanometer. This accuracy is controlled by the intensity of the light source, the width of the cuff, and the size of the aperture, and also by the rate of lowering the sphygmometer cuff pressure. The intensity of light should be 10 to 35 foot-candles, when a Weston meter is held one inch from the source of light. The light should be directly over the aperture between one-half and one inch from the foot, so that the rays of light are perpendicular to the foot. The aperture in the foot holder which permits light through to the photocell can be from one-eighth to one-half inch in diameter. Interchangeable foot holders with holes of varying diameters have been used advantageously. The size of the cuff has been discussed.

*Source of Light.*—The light on the foot should be of a "heatless" type or transferred over the foot by a Lucite rod to eliminate heating the foot. A small 3.0 volt "lens type" "penlight" bulb has also been found convenient.

*Taking the Pressure.*—The rat is placed in the rat holder and allowed to remain for a minute or two with the two hind feet resting on the table or some other solid object. The cage should be clamped in a position so that the rat's body forms a 45- to 60-degree— $\angle$ —angle with the table (Fig. 3).

The cuff is then wound around the area just above the ankle and held there by wrapping the ribbon around the cuff and clamping it to the catheter tubing with a "light pressure" clamp so as not to constrict the catheter tubing. This cuff should be kept well powdered with talc. The cuff should be applied rather loosely (but not too loosely) since the venous pressure must not be exceeded or circulation will be stopped and no change in volume of the foot will occur and, therefore, there will be no reading. If no reading is obtained, the cuff should be rewound.

The rat's foot is placed in position in the foot holder and the extinguished light is brought directly over the back of the foot which lies directly over the hole in the foot holder. The instrument is turned on, and the circuit resistance control is adjusted so that the microammeter shows that the photocell is being activated. The cuff (attached to the sphygmometer) is inflated to 250 mm., at which point the microammeter is adjusted to a reading of 150 microamperes by using a small rheostat on the light source. Small jumps or fluctuations on the



microammeter may be observed; these usually are due to movements of the foot of the rat (brought about by breathing, if the cage is not placed correctly, or by squirming) or to the interference of shadows falling on light-leaking parts of the photocell chamber. If less than ten foot-candles of light are used as the light source, shadows or reflections from the room lights onto the rat's foot will also cause erratic jumps of the microammeter needle. All extrinsic lights can be avoided by using a box over the instrument or by working in a darkened room.

The pressure is slowly lowered in the cuff at approximately 250 mm. to 0 mm. in 0.5 minute. When the systolic pressure is reached, a characteristic drop of the microammeter will be obtained. This drop will stop if the cuff is immediately inflated past the systolic pressure and will start again when the pressure in the cuff is again lowered. The microammeter will continue to approach zero

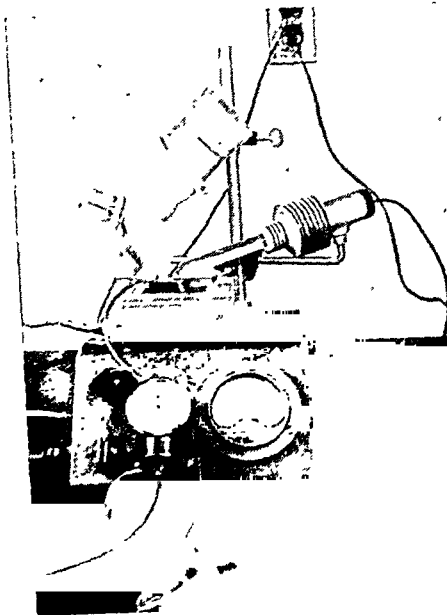


Fig. 6.—Assembled blood pressure instrument.

and may even speed up its dropping rate as pressure drops. The fall in microammeter reading is, of course, due to the change in volume of the foot incurred by blood flowing into the foot. This reduces the amount of light into the photocell.

When the pressure in the cuff gets below the venous pressure, the foot will recede to its original volume since blood can now leave the foot, and therefore the microammeter will rise to its higher reading. We have arbitrarily called this lower reading the "indicated" venous pressure, although its relationship to the actual venous pressure has not yet been established. The rate at which the cuff pressure is lowered, if too rapid, will give lower readings than if dropped more slowly, as is the case in any indirect blood pressure measurement. It has been our practice to get a range reading on the first trial by lowering the cuff

pressure at 250 mm. to 0 mm. in approximately 0.25 minute, followed by two or three readings at the standard rate of 0.5 minute for 250 millimeters. This, of course, is optional.

It has been observed that a waiting period of about one-fourth minute is necessary between readings. If no waiting period is taken, readings may not be obtained. See Fig. 6 for assembled blood pressure instrument.

#### EXPERIMENTAL

All rats received from Rockland Farms, Carworth, or our own breeding colony are placed in individual cages and numbered. In most cases no training of rats was necessary, but occasional rats were very difficult to control. As an example of the variation of normal blood pressure in these normal animals, obtained by use of the described procedures, the blood pressure of a group of seventeen rats was determined for three consecutive days. The rats varied in weight from 190 to 320 grams. The sphygmometer cuff used was 5 mm. wide and 8 cm. long, two-dip, five-inch a minute latex tubing.

The average mean determination in this first group of animals was  $118 \pm 12$  mm. for the first day,  $116 \pm 9$  mm. for the second day, and  $117 \pm 8$  mm. for the third day. (It has also been possible to take the blood pressure of rats as small as 50 grams in weight, with the appropriate foot holder and rat holder.)

#### SUMMARY

A new method and instrument for the indirect determination of blood pressure of the rat has been described. This procedure does not require the heating or the anesthetization of the animal. The principle of its functioning is based on measuring the volume change in the foot by means of a photoelectric cell before and after application of pressure by means of a miniature sphygmometer cuff applied to the ankle of the foot.

By this method the average indicated systolic blood pressure of a group of seventeen normal, unheated, unanesthetized rats was found to be 117 mm.  $\pm$  8 mm. Hg. The indicated venous pressure was found to be 18 mm.  $\pm$  4 mm. Hg.

The authors are grateful to Miss L. Lipchuck, for assistances rendered in carrying out this work, and to Mr. L. McWilliams, for the photographs.

#### REFERENCES

1. Griffith, J. Q.: Indirect Method for Determining Blood Pressure in Small Animals, *Proc. Soc. Exper. Biol. & Med.* 32: 394, 1934.
2. Byrom, F. B., and Wilson, C.: Plethysmographic Method for Measuring the Systolic Blood Pressure in the Intact Rat, *J. Physiol.* 93: 301, 1938.
3. Williams, J. R., Harrison, T. R., and Grollman, A.: A Simple Method for Determining the Systolic Blood Pressure of the Unanesthetized Rat, *J. Clin. Investigation* 18: 373, 1939.
4. Schroeder, H. A.: Arterial Hypertension in Rats, *J. Exper. Med.* 75: 513, 1942.
5. Shuler, R. H., Kupperman, H. S., and Hamilton, W. F.: Comparison of Direct and Indirect Blood Pressure Measurements in Rats, *Am. J. Physiol.* 141: 625, 1944.
6. Proskauer, G. G., Neumann, C., and Graef, I.: Measurement of Blood Pressure in Rats With Special Reference to the Effect of Changes in Temperature, *Am. J. Physiol.* 143: 290, 1945.
7. Ablondi, F., Subbarow, Y., Lipchuck, L., and Personeus, G.: Comparison of Blood Pressure Measurements in the Rat as Obtained by the Use of the Tail and Foot Methods, and by Direct Femoral Puncture, *J. LAB. & CLIN. MED.* 32: 1099, 1947.
8. *Electronics*, June, 1936, p. 38.

# COMPARISON OF BLOOD PRESSURE MEASUREMENTS IN THE RAT AS OBTAINED BY USE OF THE TAIL AND FOOT METHODS AND BY DIRECT FEMORAL PUNCTURE

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A NEW method for the indirect determination of the blood pressure of the rat has been described.<sup>1</sup> This procedure has obviated the necessity of prewarming or anesthetizing the animal, as compared with the previously described methods in the literature.<sup>2-7</sup> It became of extreme interest to compare the blood pressures obtained by this method with the various methods under similar environmental conditions.

## EXPERIMENTAL

*The Tail Instrument.*—All determinations of blood pressure made on the tail were carried out using the same type of photocell-microammeter system as described for the foot.<sup>1</sup> The change in volume of the tail was measured by the decrease or increase of light entering the photocell from a slit formed between the upper surface of the tail and a fixed metal shield before and after constriction of the tail with a sphygmometer cuff (Fig. 1). (In measuring the volume change of the foot, the amount of light going through the foot was measured before and after constriction of the ankle with a sphygmometer cuff.) This instrument gave sensitivities to volume changes on the order of, or greater than, the instrument of Williams and co-workers<sup>4</sup> as modified by Shuler and associates<sup>5</sup> or Proskauer and associates.<sup>6</sup> Its sensitivity was also of the same order as the machine used on the foot throughout all the described experiments. The size of the cuff used on the tail was 9 mm. wide by 9 cm. long, dipped in the same manner as the sphygmometer cuff used on the foot and, therefore, of the same thickness of rubber. The cuff was wound around the base of the tail and held there by wrapping a ribbon 15 mm. wide around the cuff. The indirect blood pressure was taken in exactly the same manner as described for the Williams method except that the change in volume in the tail before and after constriction with the sphygmometer cuff was observed by the increase or decrease in light entering a photocell system rather than by a water plethysmograph.

*The Foot Instrument.*—The foot instrument has been described previously.<sup>1</sup>

*The Direct Puncture Manometer.*—The instrument used for the direct measurement of blood pressure was a modification of Hamilton's optical manometer.<sup>8</sup> Instead of using the diaphragm type of mechanism, our mirror was mounted on the end of a copper-beryllium Bourdon tube. The cross-sectional diameter of the tube used was approximately three-eighths inch, while its circumferential diameter was one and one-half inches.\* The volume liquid displacement for the complete system (needle to copper tube, one-eighth inch O.D., to pet cock to Bourdon tube), between 0 and 300 mm. Hg pressure, was 0.1 cubic centimeter. By this system a linear relationship between pressure and mirror movement could be maintained from 0 to 300 mm. Hg pressure. Although the sensitivity of this method was much lower than that obtained by Hamilton's manometer, accuracy was maintained even at the higher blood pressure levels. The indicated pulse pressures of normal nembutalized rats were usually of the order of 5 to 15 mm. when pressures were taken from the femoral artery using a 23-gauge needle as a cannula.

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\*United States Gauge, New York, N. Y.

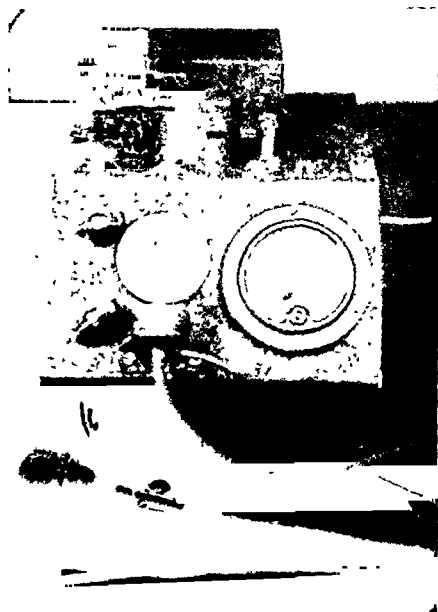


Fig. 1.—Volume change indicator adapted for tail.

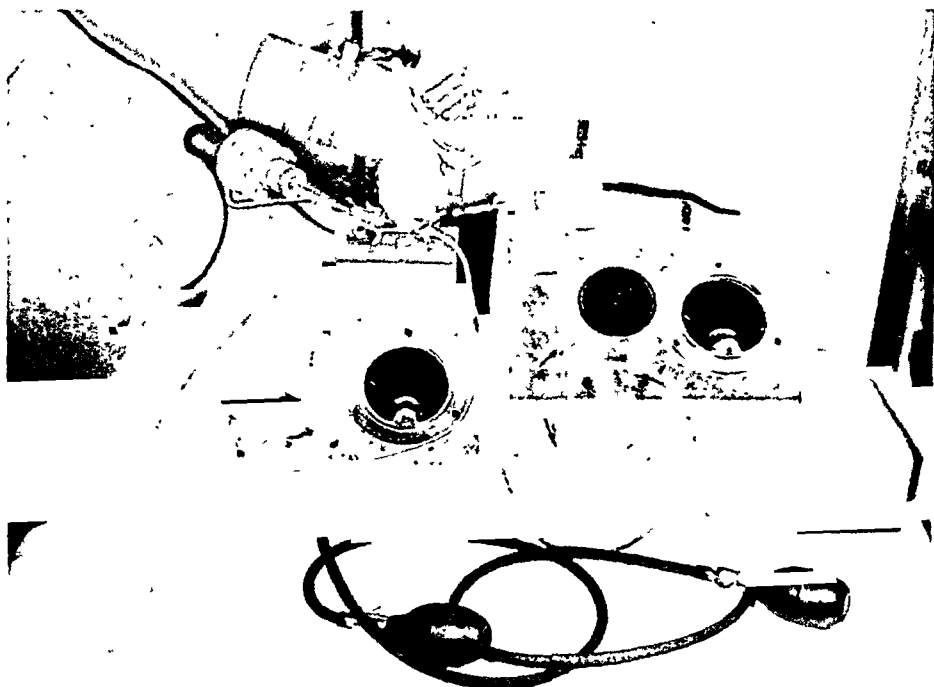


Fig. 2.—Tail and foot volume change indicators arranged for simultaneous measurement of blood pressure.

## RESULTS

*Deviations of Observed Readings in Normal Rats on the Same Day and on Different Days Under Normal and Heated Conditions by Use of Tail Method.*—Six rats were trained and the blood pressure was measured from the tail without warming the animal. Immediately after attempts were made to determine the blood pressure, the rat was placed in a carton container heated to the appropriate temperature by electric lamps. At the desired time it was removed from the carton box, and the blood pressure was again measured. The animal, as indicated in Table I, was allowed to remain at room temperature for the desired time, the blood pressure was determined, and the rat was inserted into the next carton box of desired temperature. The readings obtained are charted in Table I.

These results indicate:

1. The general unresponsiveness (no apparent volume change in the tail) of normal unanesthetized rats unless heated.

TABLE I. DEVIATION OF BLOOD PRESSURE IN TAIL OF NORMAL ANIMALS ON SAME DAY AND ON DIFFERENT DAYS UNDER NORMAL AND HEATED CONDITIONS

	DATE	ROOM TEMP.; NO HEAT	42° 3 MIN.	AL- LOWED TO COOL AT ROOM TEMP.	42° 5 MIN.	ROOM TEMP.	45° 5 MIN.	ROOM TEMP.	47° 3 MIN.	ROOM TEMP.	50° 3 MIN.	ROOM TEMP.
1	6/13	0	—	—	—	—	—	—	—	—	145/45	80/35
	6/14	0	125/30	110/25	—	—	—	—	150/50	110/25	152/50	120/50
	6/18	0	95/20	0	115/30	100/30	120/30	80/30	—	—	—	—
	6/19	0	85/20	0	—	—	115/30	60/30	—	—	120/50	85/20
2	6/13	0	—	—	—	—	—	—	—	—	145/50	110/25
	6/14	0	0	0	—	—	—	—	110/30	0	90/50	90/30
	6/18	0	0	0	95/30	60/30	90/30	70/30	140/30	80/25	—	—
	6/19	0	60/15	0	—	—	70/20	60/20	—	—	70/50	60/20
3	6/13	0	—	—	—	—	—	—	—	—	130/50	0
	6/14	0	140/45	—	—	—	—	—	145/40	118/20	135/50	130/35
	6/18	0	110/40	0	125/60	76/40	125/45	70/30	120/30	70/22	—	—
	6/19	0	50/20	0	—	—	98/45	—	—	—	135/60	95/25
4	6/13	0	—	—	—	—	—	—	—	—	110/50	0
	6/14	0	—	—	—	—	—	—	—	—	—	—
	6/18	0	110/40	0	100/25	—	100/40	0	—	—	—	—
	6/19	0	0	—	0	—	60/30	0	—	—	—	—
5	6/13	0	—	—	—	—	—	—	—	—	100/40	0
	6/14	0	—	—	—	—	—	—	—	—	—	—
	6/18	0	60/20	0	130/25	0	100/30	0	—	—	—	—
	6/19	0	0	—	—	—	125/30	60/20	—	—	—	—
6	6/13	0	—	—	—	—	—	—	—	—	110/50	80/20
	6/14	—	—	—	—	—	—	—	—	—	—	—
	6/18	0	122/14	0	130/25	0	100/35	—	—	—	—	—
	6/19	0	120/35	0	110/40	0	—	—	—	—	—	—

0. No reading.

\*. Poor reading (poor swing) due to small volume change.

-. Not done.

TABLE II. COMPARISON OF SIMULTANEOUSLY OBSERVED BLOOD PRESSURE BY USE OF TAIL AND FOOT METHOD IN UNHEATED, HEATED AND COOLED, NORMAL AND OPERATED ANIMALS

TIME	A TRAINED NORMAL RAT 50				B TRAINED OPERATED RAT 68				C TRAINED OPERATED RAT 29				D HYPERTENSIVE RAT 34			
	FOOT	TAIL	FOOT	TAIL	FOOT	TAIL	FOOT	TAIL	FOOT	TAIL	FOOT	TAIL	FOOT	TAIL	FOOT	TAIL
0	110/20	0	130/20	0	130/20	0	130/25	110/30	130/25	110/30	170/40	145/25	170/40	145/25		
2	110/20	0	130/20	0	Heat applied	←	Heat applied	←	Heat applied	←	Heat applied	←	Heat applied	←	Heat applied	←
4	110/20	0	125/20	0	←	←	160/50	145/40	160/50	145/40	175/40	150/35	175/40	150/35		
6	110/20	0	←	←	←	←	190/80	160/50	190/80	160/50	←	←	←	←		
8	115/20	75/?	135/50	130/50	←	←	185/80	175/60	185/80	175/60	←	←	←	←		
10	130/30	95/?	←	←	←	←	185/80	180/60	Heat removed	→	Heat removed	→	Heat removed	→		
12	140/60	115/35	←	←	←	←	170/70	170/70	←	←	165/40	170/20	165/40	170/20		
14	150/60	135/40	←	←	←	←	←	←	←	←	170/35	170/35	170/35	170/35		
16	145/60	135/40	←	←	←	←	←	←	←	←	←	←	←	←		
18	140/55	135/40	←	←	←	←	←	←	←	←	←	←	←	←		
20	125/60	130/50	←	←	←	←	145/50	160/60	Heat removed	→	Cage cooled	→	170/35	170/35		
22	120/30	110/40	←	←	←	←	145/50	160/50	Cage cooled	→	←	←	←	←		
24	←	←	130/40	150/55	←	←	130/40	150/55	←	←	←	←	←	←		
26	110/25	65/?	125/30	120/20	←	←	145/60	130/35	145/60	130/35	←	←	←	←		
28	110/20	?	←	←	←	←	145/80	120/30	145/80	120/30	180/35	180/35	180/35	180/35		
30	110/20	?	125/30	120/20	←	←	←	←	←	←	195/90	180/35	195/90	180/35		
32	110/20	0	125/30	90/?	←	←	135/20	115/30	135/20	115/30	←	←	←	←		
34	←	←	125/30	60/?	←	←	←	←	←	←	205/90	205/90	205/90	205/90		
36	110/20	0	←	←	←	←	←	←	←	←	←	←	←	←		
38	←	←	←	←	←	←	135/20	115/30	135/20	115/30	←	←	←	←		
40	←	←	←	←	←	←	135/20	110/30	135/20	110/30	175/70	165/35	175/70	165/35		
42	←	←	125/30	0?	←	←	←	←	←	←	170/40	145/40	170/40	145/40		
44	←	←	←	←	←	←	←	←	←	←	170/40	145/40	170/40	145/40		

0, No readings obtainable.

?, Not done.

2. The wide variation of "normal" blood pressure of normal animals when heated in the same way on different days.

3. The wide variation of response to given temperature changes of the same rat on different days.

*Comparison of Simultaneously Observed Blood Pressures by Use of Tail and Foot Methods in Unheated, Heated and Cooled, Normal and Operated Animals.*—Each animal used was placed in the rat holder adapted for foot blood pressure determination so that readings could be made simultaneously on the tail and on the foot (see Fig. 2). When warmed, the animals were heated by means of a portable heat lamp, so adjusted as not to reflect or shine directly into the apertures of the photocells of either machine.

The cuffs used in the tail method and the foot method were of such a diameter that they were proportional to the size of the appropriate appendage, that is, for the tail cuff, a 9 mm. wide, 9 cm. long cuff was used, while on the foot, where the cross-sectional diameter is smaller, a 5 mm. wide 7 cm. long cuff was used. Both cuffs were of the same thickness rubber drawn in the same speed from the same Latex (two-dip, 5 inches a minute).

In all cases, readings recorded were the average either of two or three readings. Sensitivities of both machines to volume change were the same. The results obtained in four rats picked at random are given in Table II.

These four experiments, which are a few of many such trials, indicate the following:

1. Not all rats gave volume changes in the tail when normal (unheated). In all cases (approximately 10,000 readings) blood pressure recordings (volume changes) were and have been obtained in using the foot method without heating the animal.

2. When warmed, those animals which gave no detectable volume change in the tail gradually gave increasingly larger volume changes in the tail at higher and higher blood pressure levels. When cooled by packing the metal portion of the cage with a cold water bag or ice, readings and volume changes gradually diminished until both were lost.

3. Readings were obtained continuously by use of the foot method with a definite rise of blood pressure due to heating. An increase of volume change was also observed. Heated rats are therefore no longer "normal" animals in reference to blood pressure.

4. The indicated blood pressure in the tail is not necessarily related to the indicated blood pressure in the foot in unheated unanesthetized animals, although in the heated animals the indicated blood pressures of both methods agree within the experimental error of the methods.

*Comparison of Observed Readings by Direct Femoral Puncture and by Tail and Foot Methods in Nembutalized Animals, and the Effect of Nembutal on "Vasoconstrictor Reflex."*—Three previously trained animals were used in this experiment. Before anesthetization, the blood pressure was measured both by the tail procedure and by the foot procedure simultaneously. The animal was then anesthetized intraperitoneally with nembutal, 5 mg. per 100 grams, and the blood pressure was followed as the anesthetic took effect. After anesthesia

TABLE III. CORRELATION OF FOOT METHOD AND TAIL METHOD BEFORE ANESTHESIA, AND CORRELATION OF BOTH METHODS WITH DIRECT PUNCTURE UNDER ANESTHESIA

NORMAL RAT 17					NORMAL RAT 19					TRAINED OPERATED RAT 71				
TIME (MIN.)	DIRECT	FOOT	TAIL		TIME (MIN.)	DIRECT	FOOT	TAIL		TIME (MIN.)	DIRECT	FOOT	TAIL	
0	-	90/20	0	Unanesthetized	0	-	125/15	80/20	Unanesthetized	0	-	110/30	0	Unanesthetized
3	-	90/20	0	Nembutalized	2	-	125/20	80/20	Nembutalized	5	-	110/30	0	Nembutalized
9	-	95/30	50/20	→	8		155/40	125/20	→	42		130/25	95/20	→
15	-	115/30	75/30		17		125/25	90/20		44		135/25	95/20	
26	90/	85/20	80/15		27	120	125/20	110/20		45		140/25	95/25	
30	90/	95/20	85/20		42	120	125/20	110/20		54	140	145/20	100/25	
										57	140	145/20	100/25	0.1 c.c. hypertensin
										59	180	175/35	140/30	←
										59½	185	190/40	160/40	
										59½	185	195/35	195/40	
										59½	185	185/40	185/50	
										60	140	135/30	160/50	
										125	125	125/30	130/25	
										61	130	125/30	115/20	
										61½	125	125/30	110/25	
										62	125	125/30	110/25	

0, No readings obtainable.

-, Not done.



was complete (third stage), the blood pressures were taken and the direct puncture instrument was then cannulated to the femoral artery on the right leg. The left foot was used to follow the blood pressure by the foot technique, while simultaneously observing the blood pressure in the tail.

In these three animals, obstruction of the upper respiratory passages due to the anesthetic used<sup>7</sup> was not encountered, and tracheotomy was not necessary.

In these three animals, the effect of anesthesia on the vasoconstrictor reflex is demonstrated (Table III). In the third animal various substances (pressor or depressor) were injected into the femoral vein and the blood pressure was followed by all three methods.

The results of these experiments indicate:

1. Normal unheated unanesthetized rats which gave no volume changes in the tail (and, therefore, presumably no blood pressure) when anesthetized with nembutal gradually gave volume changes in the tail as the degree of anesthesia increased without application of heat to the animal. It is, therefore, concluded that both anesthetization and heating of the animal have the same effect on the "vasoconstrictor reflex" in regard to the volume change and the indicated blood pressure in the tail. It therefore becomes apparent why agreement between the tail method and the direct puncture method is obtained in the anesthetized animal,<sup>5,7</sup> but it does not necessarily mean that there is agreement between the indicated blood pressure using the tail method and the systemic blood pressure in the unanesthetized animal.

2. Agreement of indicated blood pressures obtained by the foot method, tail method, and direct puncture method in the anesthetized animal was within the limits of the experimental errors.

#### SUMMARY

1. A comparison of indicated blood pressures as obtained by use of the foot and the tail has been made in both normal unheated and operated unheated animals. The agreement of the values obtained has been very poor, although both methods depend on the same principle for determination of blood pressure; that is, the change in volume in the appropriate appendage is observed by means of a photocell system before and after constriction of a sphygmometer cuff applied to the specific appendage.

2. The blood pressure of normal unheated rats as measured by the tail method has varied between 50 and 130 mm. where a volume change in the tail occurred. (Proskauer and co-workers<sup>6</sup> have reported a value of 65 to 95 mm. of Hg.) In very normal unheated animals, no volume change and, therefore, presumably no blood pressure could be detected. In all cases volume changes were observed when using the foot. The average blood pressure obtained in a group of seventeen normal unheated rats picked at random was  $117 \pm 8$  mm. Hg.<sup>1</sup> This value is in good agreement with those obtained by direct puncture as reported by Schroeder<sup>7</sup> for normal anesthetized rats.

3. It was observed that in the tail of the normal unanesthetized animal, in cases where there was no volume change unless heated, the volume change gradually increased with increased heat applied to the rat, and that these

volume changes occurred at concurrently higher blood pressure levels. Attempts to standardize the amount and the rate of heating applied to the rat were made with no success. By using the foot method, readings were obtained without the application of heat. In the foot, as heat was applied, the volume change also increased but at a much lower rate, while the blood pressure rose from the level obtained in the unheated normal animal. It is, therefore, apparent that normal heated animals are no longer "normal" in regard to blood pressure. It is also apparent that the blood pressure in the tail, as indicated by the volume change principle, is not directly related to the systemic blood pressure in the unheated unanesthetized rat, although under abnormal conditions (heating or anesthesia) a relationship between indicated foot blood pressures and the indicated tail blood pressures was obtained.

4. Under the influence of anesthesia volume changes in the tail could be easily obtained without the application of heat, and agreement of all three methods was fairly good when simultaneous blood pressure readings were taken by tail, foot, and direct puncture.

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#### REFERENCES

1. Kersten, H., Brosene, W. G., Ablondi, F., and Subbarow, Y.: A New Method for the Indirect Measurement of Blood Pressure in the Rat, *J. LAB. & CLIN. MED.* 32: 1090, 1947.
2. Griffith, J. Q.: Indirect Method for Determining Blood Pressure in Small Animals, *Proc. Soc. Exper. Biol. & Med.* 32: 394, 1934.
3. Byrom, F. B., and Wilson, C.: A Plethysmographic Method for Measuring the Systolic Blood Pressure in the Intact Rat, *J. Physiol.* 93: 301, 1938.
4. Williams, J. R., Harrison, T. R., and Grollman, A.: A Simple Method for Determining the Systolic Blood Pressure of the Unanesthetized Rat, *J. Clin. Investigation* 18: 373, 1939.
5. Shuler, R. H., Kupperman, H. S., and Hamilton, W. F.: Comparison of Direct and Indirect Blood Pressure Measurements in Rats, *Am. J. Physiol.* 141: 625, 1944.
6. Proskauer, G. G., Neumann, C., and Graef, I.: Measurement of Blood Pressure in Rats With Special Reference to the Effect of Changes in Temperature, *Am. J. Physiol.* 143: 290, 1945.
7. Schroeder, H. A.: Arterial Hypertension in Rats, *J. Exper. Med.* 75: 513, 1942.
8. Hamilton, W. F., Brewer, G., and Brotman, I.: Pressure Pulse Contours in the Intact Animal, *Am. J. Physiol.* 107: 427, 1934.

# THE DIAGNOSIS OF PANCREATIC DISEASE BY ENZYME TESTS

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THE clinician has long found the diagnosis and treatment of diseases of the pancreas to be among the most difficult and uncertain of all diseases. This has been adjudged partly to be due to (1) the lack or obscurity of symptoms in early or moderately advanced stages of pancreatic disease clinically, and (2) the complexity of pancreatic tests and their impracticability for the practicing physician, biochemically.

In this paper I report the results of studies carried out from a series of patients seen in gastroenterologic practice which have been used as an aid in the diagnosis and treatment of diseases of the pancreas. This study was undertaken with the view of utilizing some procedure which would aid in establishing a diagnosis of pancreatic disease.

The American Gastroenterological Association<sup>1</sup> appointed a committee to study the pancreatic problem. Among other reports, two extensive investigations for the committee were published by two groups of investigators, one from the laboratories of the Mayo Clinic,<sup>2</sup> and one from the laboratories of the Western Reserve University School of Medicine and Hospital.<sup>3</sup>

I therefore believed it might be of interest to describe additional pancreatic studies in gastrointestinal diseases in the attempt to explore further the practicability and reliability of pancreatic diagnostic procedures.

These studies do not touch upon acute pancreatitis, which is in the nature of an acute surgical emergency, but rather on diseases of the pancreas such as cancer, stone, and chronic inflammation, as well as on disturbances of the pancreas caused by primary diseases of contiguous organs.

Extensive evidence has now been accumulated by surgeons and pathologists alike<sup>4</sup> that dyspepsia is often the primary or secondary expression of chronic pancreatitis. For example, Rich and Duff found "repeated focal necrosis, focal hemorrhages, and epithelial metaplasia" in routine pancreatic examinations during autopsies at the Johns Hopkins Hospital.<sup>5</sup> These findings have been duplicated by other observers.<sup>6</sup> Osler also drew attention to this fact and stated that the confusion existing on the subject was due to the "personal equation of the laboratory workers."<sup>7</sup>

Clinicians attempting an analysis of the role of chronic pancreatitis in causing dyspepsia are represented by Koehler,<sup>8</sup> who found that 26 per cent of his series of patients suffering from "functional indigestions" showed duodenal amylase deficiency. Rivers<sup>9</sup> survey suggests a similar conclusion.

<sup>1</sup>From the Departments of Internal Medicine and Experimental Medicine, College of Medical Evangelists.

<sup>2</sup>Presented before the meeting of the Western Society for Clinical Research, San Francisco, Nov. 2, 1946.

<sup>3</sup>Received for publication, July 31, 1946.

Although pancreatic stones are rarely diagnosed by the clinician during life, the studies of Ludin<sup>10</sup> have shown that routine x-ray examinations of 542 pancreases correlated with histologic study revealed concretions in 5 per cent of the cases. These concretions varied in size from the head of a pin to that of a hazelnut. Snell and Comfort,<sup>11</sup> Moss and Freis,<sup>12</sup> and others have shown the value of pancreatic functional tests in patients with pancreatic lithiasis.

In reference to the condition described as "pancreatic edema," many surgeons have described the frequency of this condition at operation.<sup>13</sup> Archibald in 1913 was one of the first surgeons to report a series of patients suffering from attacks of acute epigastric pain in whom operation revealed only pancreatic edema.<sup>14</sup> Pratt,<sup>15</sup> a lifelong worker in pancreatic diseases, described the accumulative evidence which indicates that attacks of epigastric pain are due, not infrequently, to mild acute pancreatic lesions.

Experienced surgeons like Elman,<sup>16</sup> Cole,<sup>13b</sup> Demel,<sup>17</sup> Loeffler,<sup>18</sup> and others too numerous to mention all have reported series of patients suffering from attacks of epigastric pain in whom careful surgical exploration revealed diffuse edema of the pancreas, without evidence of necrosis or hemorrhage.

Pratt<sup>15</sup> has emphasized the fact that in patients where the symptoms are indicative of gall bladder disease, or where the clinical picture is that of gallstones and the cholecystograms are negative, pancreatic disease is frequently present.

The need for study of pancreatic disorders has thus been keenly felt for some time.

Since Einhorn's introduction of the duodenal bucket in 1910<sup>20</sup> numerous investigators<sup>21-26</sup> have reported the results of their procedures in the testing of pancreatic function. However, the recent studies of Free and Myers<sup>26</sup> present such a comprehensive and improved system of technique that I have used their methods in testing pancreatic function.

#### METHOD

A series of eight subjects in good health who were free of any gastrointestinal signs or symptoms were selected for normal controls. They consisted of five women and three men ranging from 20 to 58 years of age. Duodenal drainages and tests were routinely performed in all subjects on an empty stomach in the morning. The fasting duodenal secretion was first extracted and then one ounce of warm olive oil was injected through the tube into the duodenum and retained for ten minutes. The duodenal contents were siphoned off in twenty-, forty-, and sixty-minute periods after the olive oil stimulation and each specimen was examined for proteinase, amylase, and lipase according to the methods devised by Free and Myers.<sup>26</sup> Thus twelve pancreatic functional determinations were performed at each duodenal drainage. As described in Tables I to VI, the number of duodenal drainages varied in each patient from one to five, giving a range of functional tests numbering from twelve to sixty for each subject. In all, a total of more than one thousand duodenal pancreatic tests are herein reported. Bile pigment in each diluted sample was determined by the icterus index.

#### CASE MATERIAL AND RESULTS

Table I shows that in over 288 pancreatic enzyme determinations taken from eight normal subjects, the average values for the fasting and twenty-, forty-, and sixty-minute periods of stimulation by olive oil were: proteinase,

3.6 mg.; amylase, 7.5 mg.; and lipase, 8 milliliters. However, it is noted that the normal variations for proteinase, amylase, and lipase are great. They range from 0.6 to 7.4 mg. in proteinase, from 0 to 17.3 mg. in amylase, and from 0 to 31.4 ml. in lipase. In these normal subjects zero readings were not found again at subsequent tests.

TABLE I. DUODENAL CONTENT ANALYSES FOR PANCREATIC ENZYMES IN EIGHT NORMAL SUBJECTS OVER TWENTY-FOUR TEST PERIODS; AVERAGE VALUES, 288 TESTS

ENZYME	FASTING	20 MIN. AFTER OLIVE OIL STIM- ULATION	40 MIN. AFTER OLIVE OIL STIM- ULATION	60 MIN. AFTER OLIVE OIL STIM- ULATION	MINIMUM VARIATION	MAXIMUM VARIATION
Proteinase*	2.5	3.0	4.2	4.9	0.6	7.4
Amylase†	5.2	6.4	8.2	10.1	0	17.3
Lipase‡	4.8	5.7	6.8	15.0	0	31.4

\*Milligrams tyrosine equivalent.

†Milligrams glucose equivalent.

‡Milliliters 0.05 N sodium hydroxide equivalent.

Table II shows that in over sixteen pancreatic enzyme determinations taken from eight subjects with "functional" or nonorganic disturbances of the upper gastrointestinal tract, the average values for the fasting and twenty- and sixty-minute stimulation intervals were: proteinase, 2.0 mg.; amylase, 4.3 mg.; and lipase, 4.0 milliliters. The individual variations in "functional" disorders are considerable. They range from 0.2 to 3.8 mg. in proteinase, from 0.6 to 8.5 mg. in amylase, and from 0 to 8.5 ml. in lipase. Zero findings were not repeated at subsequent determinations. These eight subjects were diagnosed as having upper digestive tract dyspepsia, six from gastrointestinal neuroses and two from the postcholecystectomy syndrome.

TABLE II. DUODENAL CONTENT ANALYSES FOR PANCREATIC ENZYMES IN EIGHT PATIENTS WITH "FUNCTIONAL" OR NONORGANIC DISORDERS OF THE UPPER GASTROINTESTINAL TRACT; AVERAGE VALUES FOR SIXTEEN TESTING PERIODS, 192 TESTS

ENZYME	FASTING	20 MIN. AFTER OLIVE OIL STIM- ULATION	40 MIN. AFTER OLIVE OIL STIM- ULATION	60 MIN. AFTER OLIVE OIL STIM- ULATION	MINIMUM VARIATION	MAXIMUM VARIATION
Proteinase*	1.3	1.8	2.2	2.7	0.2	3.8
Amylase†	2.9	4.0	4.9	5.6	0.6	8.5
Lipase‡	2.9	3.4	4.5	5.1	0	8.5

\*Milligrams tyrosine equivalent.

†Milligrams glucose equivalent.

‡Milliliters 0.05 N sodium hydroxide equivalent.

Table III shows that in twenty subjects suffering from organic diseases of the upper digestive tract, the average values for all the intervals previously described were: proteinase, 1.3 mg.; amylase, 3.7 mg.; and lipase, 3.6 milliliters. The individual test readings for enzymes here also are very variable. The range for proteinase is from 0 to 5.6 mg.; for amylase, from 0 to 10.4 mg.; and for lipase, from 0 to 13.6 milliliters. In contrast with the normal controls and the "functional" cases, if zero readings were obtained and found again at subsequent tests, extensive involvement of the pancreas by cancer was present as occurred in the three cases of cancer of the pancreas (Table V, Cases

TABLE III. DUODENAL CONTENT ANALYSES FOR PANCREATIC ENZYMES IN TWENTY PATIENTS WITH ORGANIC DISEASES OF THE UPPER GASTROINTESTINAL TRACT; AVERAGE VALUES FOR FORTY-THREE TESTING PERIODS, 510 TESTS

ENZYME	FASTING	20 MIN. AFTER OLIVE OIL STIM- ULATION	40 MIN. AFTER OLIVE OIL STIM- ULATION	60 MIN. AFTER OLIVE OIL STIM- ULATION	MINIMUM VARIATION	MAXIMUM VARIATION
Proteinase*	0.7	1.2	1.5	1.7	0	5.6
Amylase†	2.2	3.0	3.4	6.1	0	10.4
Lipase‡	0.9	3.0	4.8	5.7	0	13.6

\*Milligrams tyrosine equivalent.

†Milligrams glucose equivalent.

‡Milliliters 0.05 N sodium hydroxide equivalent.

3, 4, and 18). If near zero readings were consistently obtained, particularly in proteinase, extensive disease of the pancreas was found such as in the three cases of pancreatic cirrhosis with or without lithiasis (Table V, Cases 2, 6, and 7).

#### COMMENT

The twenty patients with organic disease listed in Tables III and VI consisted of three patients with carcinoma of the pancreas, two with cholithiasis, one with choledocholithiasis, one with pancreatic lithiasis, two with pancreatic cirrhosis (diagnosed at operation), three with cirrhosis of the liver, three with duodenal ulcer, one with gastric ulcer, and one with infectious hepatitis and cholangitis, carcinoma of the pyloric end of the stomach, carcinoma of the gall bladder and bile ducts, and nontropical sprue, respectively.

Table IV compares the total average enzyme values for all interval readings found in normal subjects with those of functional and organic diseases of the upper digestive tract. It is seen that the respective average readings in the "normal" controls and the "functional" cases appear to be clearly defined. However, the difference between the average readings of the "functional" and those of the "organic" diseases seems small. This lack of contrast appears to be due to the inclusion of a group of cases under "organic" causes in which the pancreas itself was not primarily involved. Cases of gastric and duodenal ulcer or cholelithiasis are examples. On the other hand, when the cases in which the pancreas is involved primarily, such as by carcinoma or cirrhosis or the stone, are examined the test readings are consistently zero or very close to zero. The minimal and maximal ranges varied quite widely in any one patient in whom the pancreas was not extensively and markedly involved and rendered it impossible to determine accurately the category in which one reading placed a patient.

Percentages were not calculated since the range of variation in (1) normal subjects, (2) patients with organic disease of the upper digestive tract, and (3) patients with functional diseases of the upper digestive tract are so great that only repeated zero readings or repeated near zero readings are of diagnostic significance.

One single determination of the proteinase, amylase, or lipase values would appear to be adequate since the enzyme values were consistent in rendering the same information in one patient. This was particularly true where severe

TABLE IV. DUODENAL CONTENT ANALYSES FOR PANCREATIC ENZYMES; TOTAL AVERAGE OF FOUR ENZYME READINGS IN COMPARATIVE STUDIES OF UPPER GASTROINTESTINAL TRACT DISEASES

ENZYME	NORMAL CONTROL SUBJECTS	FUNCTIONAL OR NONORGANIC DISEASES	ORGANIC DISEASES
Proteinase*	3.6	2.0	1.3
Minimum variation	0.6	0.2	0
Maximum variation	7.4	3.8	5.6
Amylase†	7.5	4.3	3.7
Minimum variation	0	0.6	0
Maximum variation	17.3	8.5	10.4
Lipase‡	8.0	4.0	3.6
Minimum variation	0	0	0
Maximum variation	31.4	8.5	13.6

\*Milligrams tyrosine equivalent.

†Milligrams glucose equivalent.

‡Milliliters 0.05 N sodium hydroxide equivalent.

TABLE V. ENZYME VALUES IN EXTENSIVE ADVANCED PANCREATIC DISEASE PROVED AT OPERATION

CASE	DIAGNOSIS	NUMBER OF TESTS	FASTING			20-MIN. STIMULATION			40-MIN. STIMULATION			60-MIN. STIMULATION		
			P.	L.	A.	P.	L.	A.	P.	L.	A.	P.	L.	A.
3	Carcinoma of head of pancreas	1	0	0	0	0			0			0		
		2	0	0	0	0			0			0		
2	Advanced pancreatic cirrhosis	1	0	0	0	0.2	0.7	0.5	0.5	1.8	0.8	0.5	2.4	1.0
		2	0	0	0	0.4	1.5	1.1	0.4	1.7	1.3	0.4	2.1	1.0
		3	0.2	0.8	1.5	0.2	1.3	1.2	0.5	2.1	1.0	0.3	1.5	1.3
		4	0	0	0	0	0.5	0.3	0.3	0.9	0.9	0.4	1.5	1.0
		5	0.5	1.9	1.0	0.5	1.4	1.0	0.5	1.8	1.3	0.6	2.4	1.0
4	Carcinoma of head and body of pancreas	1	0	0	0	0	0	0	0	0	0	0	0	0
		2	0	0	0	0	0	0	0	0	0	0	0	0
7	Advanced cirrhosis of the liver and pancreas	1	0	0	0	0.2	0.8	0.7	0.3	1.8	0.8	0.2	3.2	1.0
		2	0	0	0	0.2	1.5	0.3	0.2	1.9	0.8	0.1	2.6	1.1
18	Carcinoma of head and body of pancreas	1	1	0	0	0	0	0	0	0	0	0	0	0
		2	2	0	0	0	0	0	0	0	0	0	0	0
6	Extensive pancreatic lithiasis with cirrhosis	1	0	0.8	0.3	0.2	1.5	0.7	0.2	2.1	0.3	0.3	2.4	0.4

P., Proteinase; L., lipase; A., amylase.

TABLE VI. AVERAGE ENZYME VALUE IN ORGANIC DISEASES OF THE UPPER DIGESTIVE TRACT

CASE	DIAGNOSIS	NUMBER OF TESTS	PROTEINASE	AMYLASE	LIPASE
1	Cholelithiasis, cholecystitis	12	1.1	2.2	3.2
5	Carcinoma, gall bladder	4	0.9	2.6	3.3
8	Cirrhosis of liver	12	2.95	4.9	7.2
9	Nontropical sprue	4	3.1	7.1	8.6
10	Cholelithiasis	8	1.2	4.5	4.5
11	Cholecystitis with lithiasis	8	1.3	6.5	3.9
12	Penetrating duodenal ulcer	8	1.2	3.0	4.5
13	Chronic duodenal ulcer	8	1.9	6.1	7.6
14	Chronic duodenal ulcer	8	1.5	4.2	4.2
15	Gastric ulcer	8	2.4	7.6	6.3
16	Carcinoma, pyloric stomach	8	1.2	2.6	2.5
17	Cirrhosis of liver	8	1.6	4.5	3.9
19	Chronic cholecystitis	8	1.7	2.9	2.7
20	Infectious epidemic hepatitis	8	1.7	4.2	4.4

primary disease of the pancreas was present, as in the three cases of carcinoma of the pancreas, each accompanied by jaundice.

In the three patients with pancreatic carcinoma the enzyme values were zero throughout for every proteinase, amylase, and lipase reading at the fasting and twenty-, forty-, and sixty-minute stimulation periods. In the case of marked pancreatic cirrhosis with or without lithiasis, the readings were all consistently close to zero. These two primary diseases of the pancreas, that is, cancer or extensive cirrhosis (with or without stone), therefore consistently gave findings of diagnostic value in the duodenal pancreatic enzyme tests.

#### SUMMARY

1. Over 1,000 pancreatic enzyme tests (Free and Myers<sup>20</sup>) were made from the duodenal secretions in a group of normal subjects, in a group of patients with "functional," or nonorganic disease of the upper digestive tract, and in a group of patients with organic disease of the pancreas, biliary tract, stomach, or duodenum.

2. The range and mean of duodenal pancreatic enzyme tests for normal subjects and patients suffering from upper digestive tract disorders are described. Variations of test findings are very wide in both normal subjects and patients with most organic diseases of the pancreas or upper digestive tract.

3. Normal subjects as well as patients with extensive and severe pathologic involvement of the pancreas such as cancer may give zero or near zero test readings in individual duodenal pancreatic enzyme tests.

4. In patients with organic disease of the stomach or biliary tract, pancreatic duodenal enzyme tests revealed no individual distinctive or diagnostic findings. However, their group mean value was definitely lower than the comparable value in normal subjects.

5. A group of ten patients with chronic low-grade or moderate inflammation of the pancreas, as revealed at operation, had a series of duodenal pancreatic enzyme tests performed before and after operation. The duodenal enzyme study failed to show any consistently abnormal findings of diagnostic value either pre- or postoperatively.

6. In six cases of extensive involvement of the pancreas by cancer or cirrhosis with pancreatic lithiasis, 125 tests gave abnormal findings in the duodenal pancreatic enzyme tests. These abnormal findings were either zero or near zero values.

7. If a zero reading was found in the pancreatic enzyme tests of a normal subject, it was not consistently found at subsequent determinations. In the presence of advanced and extensive pancreatic disease such as cancer or cirrhosis, zero and near zero readings were found consistently at all subsequent determinations.

#### CONCLUSION

Extensive and advanced disease of the pancreas such as cancer or cirrhosis with lithiasis gave repeated zero or near zero readings in the duodenal pan-



creatic enzyme tests. Pancreatic enzyme tests are useful in diagnosing advanced and extensive disease of the pancreas.

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## REFERENCES

1. American Gastroenterological Association: Committee Report. Survey of Enzyme Tests by Members of A.G.E. *Am. J. Digest. Dis.* 1: 462, 466, 1934-1935.
2. Comfort, M. W., Parker, R. L., and Osterberg, A. E.: The Concentration of Pancreatic Enzymes in the Duodenum of Normal Persons and Persons With Disease of the Upper Part of the Abdomen, *Am. J. Digest. Dis.* 6: 249, 1939.
3. Free, A. H., Beams, A. J., and Myers, V. C.: Studies of the Enzyme Activities of Duodenal Contents as a Means of Evaluating Pancreatic Function, *Gastroenterology* 1: 188, 1943.
4. Osler, Sir William: *The Principles and Practice of Medicine* (edited by Henry Christian), ed. 15, New York, 1944, D. Appleton-Century Company, Inc., p. 778.
5. Rich, A. R., and Duff, G.: Experimental and Pathological Studies on Pathogenesis of Acute Hemorrhagic Pancreatitis, *Bull. Johns Hopkins Hosp.* 58: 212, 1936.
6. Katsch, G., and Brinck, J.: *Handbuch der Inneren Medizin*, ed. 3, Berlin, 1938, vol. III, p. 1018, Julius Springer.
7. Osler, Sir William: Quoted by Pratt, J. H., in *Diseases of the Pancreas*, Oxford Medicine (edited by Henry Christian), New York, 1939, Oxford University Press, vol. III, part II, p. 490.
8. Koehler, A. E.: Functional Indigestion, *California & West. Med.* 48: 247, 1938.
9. Rivers, A. B.: Dangers of Treating "Indigestion" by Advertised Nostrums, *Proc. Staff Meet., Mayo Clinic* 13: 87, 1938.
10. Ludin, M.: Pankreas Entzündungen, *Arch. f. Verdauungskr.* 63: 249, 1938.
11. Snell, A. M., and Comfort, M. W.: Hepatic Lesions Presumably Secondary to Pancreatic Lithiasis and Atrophy, *Am. J. Digest. Dis. and Nut.* 4: 215-218, 1937.
12. Moss, R. E., and Freis, E. D.: Clinical Features of Pancreatic Lithiasis; Report of Two Cases, *New England J. Med.* 227: 591, 1942.
13. a. Zoepffel, H.: Precursory Stages of Acute Necrosis of the Pancreas, *Deutsche Ztschr. f. Chir.* 175: 301, 1922.  
b. Cole, W. H.: Treatment of Acute Pancreatitis, *Am. J. Surg.* 40: 245, 1938.
14. a. Archibald, E. W.: Does Cholecystenterostomy Divert the Flow of Bile From the Bile Duct? *Canad. M. A. J.* 2: 557, 1912.  
b. *Ibid.*: Ideas Concerning the Causation of Some Cases of Pancreatitis, *Canad. J. M. & S., Toronto*, 33: 263, 1913.  
c. *Ibid.*: The Experimental Production of Pancreatitis in Animals, *Surg., Gynec. & Obst.* 288: 529, 1919.
15. Pratt, J. H.: *Diseases of the Pancreas*, Oxford Medicine (edited by Henry Christian), New York, 1939, Oxford University Press, vol. III, part II, p. 506.
16. a. Elman, R.: Variations of Blood Amylase During Acute Transient Disease of the Pancreas, *Ann. Surg.* 110: 379, 1937.  
b. *Ibid.*: Acute Inflammation of the Pancreas, *Surg., Gynec. & Obst.* 61: 670, 1935  
c. *Ibid.*: Acute Interstitial Pancreatitis; Clinical Study of Thirty-seven Cases, *Surg., Gynec. & Obst.* 57: 291, 1933.
17. a. Demel, R.: Umstrittene Fragen bei akuter Pankreas-Nekrose, *Wien. Klin. Wchnschr.* 49: 1273, 1936.  
b. Fehler, Komplikationen und Störungen im Anschluss an Magenresektionen, *Arch. f. klin. Chir.* 193: 42, 1938.
18. Loeffler, W.: Ueber die Pankreas Krankheit, *Arch. f. Verdauungskr.* 63: 249, 1938.
19. Einhorn, Max: A Practical Method of Obtaining the Duodenal Contents in Man, *Med. Record* 77: 98, 1910.
20. Einhorn, M., and Rosenbloom, J.: A Study of the Duodenal Contents in Man, *Arch. Int. Med.* 6: 666, 1910.
21. Chace, A. F., and Myers, V. C.: The Examination for Diagnostic Purposes of the Enzyme Activity of Duodenal Contents, *Arch. Int. Med.* 12: 628, 1913.

22. a. Crohn, B. B.: The Diagnosis of the Functional Activity of the Pancreatic Gland by Means of Ferment Analyses of the Duodenal Contents and of the Stools, *Am. J. M. Sc.* 145: 393, 1913.  
b. Ibid.: The Chemical Examination of Duodenal Contents as a Means of Diagnosis in Conditions of Jaundice, *J. A. M. A.* 64: 565, 1915.  
c. Ibid.: Studies in Pancreatic Disease, *Arch. Int. Med.* 15: 581, 1915.
23. McClure, C. S., Wetmore, A. S., and Reynolds, Lawrence: New Methods for Estimating Enzymatic Activities of Duodenal Contents of Normal Man, *Arch. Int. Med.* 27: 706, 1921.
24. McClure, C. W.: *Functional Activities of the Pancreas and Liver*, New York, 1937, Medical Authors Publishing Co.
25. Swalm, W. A.: Exhibit on Coprology, Section on Gastroenterology, A. M. A. Convention, Atlantic City, N. J., June 12, 1935.
26. Free, A. H., and Myers, V. C.: The Estimation of the Enzymes, Amylase, Proteinase, and Lipase in Duodenal Contents, *J. LAB. & CLIN. MED.* 28: 1387, 1943.

# INTRAVENOUSLY ADMINISTERED GELATIN—A TOXICITY STUDY

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IT IS generally recognized that an infusion of blood or plasma is the most effective means of compensating for fluid loss which occurs in hemorrhage and shock of various types. Since blood and plasma are not always immediately at hand, however, it is desirable that a plasma substitute be found which is innocuous, effective, inexpensive, and readily available.

The recent literature records that substitutes somewhat less effective than plasma in restoring circulating fluid volume, but much more effective than crystalloidal solutions, are aqueous solutions of macromolecular substances, such as gelatin, gum arabic, pectin, ovalbumin, polyvinyl alcohol, and methyl cellulose. Gelatin<sup>1-20</sup> appears to possess desirable characteristics to a greater degree than most of the other macromolecular substances mentioned.

Observations in this laboratory on the toxic effects in animals of infused gelatin solutions which were subsequently tested for efficacy by laboratory animal experimentation<sup>11, 18</sup> and clinical investigation<sup>15, 16, 19, 20</sup> confirm the findings of others in many respects. Some of the undesirable properties of gelatin, however, such as impairment of the production of plasma proteins and hemoglobin,<sup>17</sup> and degenerative and proliferative arterial lesions of the sclerosing type reported by Heuper<sup>6</sup> in dogs, are apparently observed only when large amounts of highly concentrated gelatin solution are repeatedly infused into the blood stream of intact animals, thus placing a severe strain on the circulatory system. When a 5 to 8 per cent gelatin solution is infused into dogs or human subjects suffering fluid loss from hemorrhagic, traumatic, or burn shock in amounts approximately equal to the amount of fluid which has escaped, such untoward effects have not been reported.

The experiments herewith presented were designed to test whether or not the circulatory system of dogs under the stress of light anesthesia and severe blood loss could tolerate large quantities of 5 per cent gelatin administered in divided doses over a period of several weeks.

The solutions of gelatin\* were prepared by electrodialysis of osseous calcium gelatinate. The 5 per cent solutions preserved with phenyl-mercuriborate, 1:25,000, were degraded by autoclaving to obtain a mean molecular weight of 20,000 (number average). These solutions were nonantigenic and nonpyrogenic.

## EXPERIMENTAL

Nine mongrel dogs supplied with a high protein diet supplemented with raw liver were bled of 29 to 54 per cent of their estimated blood volume at weekly intervals for periods of two to thirteen weeks (average, seven weeks). The blood, aseptically removed from the

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\*Supplied by Dr. S. H. Eppstein, of these laboratories.

exposed jugular veins under light ether anesthesia, was replaced at the same site by infusion of 5 per cent gelatin solution in amounts equal to 80 per cent of the blood removed. Control animals consisted of two dogs bled just as severely as those mentioned, but with no parenteral fluid replacement, and twenty apparently healthy dogs with no treatment whatsoever.

At intervals determinations of plasma protein and gelatin concentration in plasma, urine, and aqueous tissue extracts\* were made by the method of Waters<sup>4</sup> which makes use of the fact that gelatin is not precipitated by trichloroacetic acid but is precipitated by acid tungstate. Plasma protein nitrogen was thus determined by the usual Kjeldahl technique on the trichloroacetic acid precipitate and gelatin nitrogen by difference between nitrogen contents of the acid tungstate and trichloroacetic acid precipitates. The usual methods were employed for determination of red cell volume, hemoglobin, red cell count, white cell count, and sedimentation rate.<sup>22</sup>

Extensive pathologic studies on tissues of experimental and control dogs were done.<sup>†</sup>

On one of the nine dogs, a celiotomy was performed both prior to and one week after a series of six hemorrhages and gelatin infusions. Liver tissue was removed and examined at these times. Seven weeks later this dog was sacrificed and the tissues examined. A second dog was bled and infused with gelatin five times after a celiotomy had been performed for removal of liver tissue. He was sacrificed two weeks after the last hemorrhage and infusion.

## RESULTS

Repeated massive hemorrhages and infusions of purified gelatin in dogs under the conditions described produced no notable changes in deportment or body weight (Table I). The only gross disturbance observed was the formation of thrombi at the sites of injection in some dogs which Patek and co-workers<sup>24</sup> have demonstrated to be due to the mercurial preservative employed. Histologic examination of the jugular veins at autopsy revealed no changes in the intima. Considerable scar tissue formed about these veins, presumably due to the surgery involved, and fluid regurgitated into surrounding tissue when the needle was withdrawn. Wound healing was not impaired. There was no indication of the presence of pyrogenic or antigenic material in the gelatin.

TABLE I. SHOWING THE EFFECT OF REPEATED MASSIVE HEMORRHAGES AND GELATIN INFUSIONS ON THE BODY WEIGHTS OF DOGS

DOG	NUMBER OF HEMORRHAGES AND INFUSIONS (WK.)	INITIAL BODY WEIGHT (KG.)	CHANGE IN BODY WEIGHT (KG.)	AMOUNT OF BLOOD REMOVED (ML.)	AMOUNT OF 5% GELATIN INFUSED (ML.)
2	13	12.3	+ 2.6	5,668	4,534
3	12	12.4	+ 6.4	7,366	5,893
4	3	19.8	+ 0.2	2,345	None
5	12	15.6	+ 4.8	8,531	None
6	7	15.5	+ 1.3	3,532	2,826
10	7	9.5	+ 0.8	2,250	1,800
11	7	13.4	+ 0.1	3,539	2,824
12	2	11.6	+ 2.4	905	725
13	2	10.2	+ 0.7	640	520
14	6	19.8	- 1.3	3,155	2,524
15	5	19.0	- 0.8	2,410	1,928

\*Weighed quantities of kidney and liver from both control and gelatin-infused dogs were thoroughly macerated with saline in a Waring blender. Gelatin in the supernatant fluid was determined by the method used for plasma gelatin.

†By Dr. Walter Schiller and Dr. Bruno Volk.

Examination of blood and plasma during the experimental period revealed that the changes observed in hemoglobin and plasma protein levels could be accounted for by the severe hemorrhage alone and were not markedly affected by the infused gelatin. (Tables II and III).

TABLE II. HEMOGLOBIN, RED CELL VOLUME, AND PLASMA PROTEIN NITROGEN LEVELS OF DOGS. SUBJECTED TO WEEKLY MASSIVE HEMORRHAGES BOTH WITH AND WITHOUT REPLACEMENT OF THE WITHDRAWN BLOOD BY GELATIN SOLUTION

TIME (WK.)	TEST GROUP (WITHDRAWN BLOOD REPLACED BY GELATIN)									CONTROL GROUP (NO PARENTERAL FLUID REPLACEMENT)					
	DOG 2			DOG 3			DOG 6			DOG 4			DOG 5		
	Hg. (GM. %)	CELL VOL. (%)	PPN (MG. %)	Hg. (GM. %)	CELL VOL. (%)	PPN (MG. %)	Hg. (GM. %)	CELL VOL. (%)	PPN (MG. %)	Hg. (GM. %)	CELL VOL. (%)	PPN (MG. %)	Hg. (GM. %)	CELL VOL. (%)	PPN (MG. %)
1		48.1	1,010	14.0	45.6	843	16.5	48.9	978	20.8	58.8	924	11.6	36.4	811
2	13.2	44.0	930	8.9	31.8	825	18.5	55.0	1,025	13.6	44.4	890	8.9	31.3	865
3	12.2	40.8	977	11.0	41.4	840	13.9	44.1	1,022	11.0	36.6	838	9.5	32.1	728
4	11.2	39.6	1,006	11.4	40.8	735		31.3	873				9.9	36.3	903
5	(No hemorrhage or infusion)			14.8	46.8	886	10.4	37.5	914				9.3	32.5	923
6	13.8	46.8	969	12.6	45.6	829	9.3	35.0						27.5	777
7	11.6	40.8	879	14.0	46.8	861		31.3	919				9.9	35.0	855
8	11.0	40.8	904	13.4	45.0	847							10.1	37.5	
9	10.7	39.6	992	14.6	47.5	926								30.0	817
10	11.0	40.8	904	13.4	43.8	840							9.4	35.0	876
11	9.5	35.4	908	11.6	40.6	797							9.1	33.5	
12	9.7	32.9	956	12.8	45.0	792							7.9	30.5	948
13	8.3	31.3	898												
14	9.7	36.0	889												

TABLE III. ILLUSTRATING TYPICAL RATES OF PLASMA PROTEIN REGENERATION AND DISAPPEARANCE OF PLASMA GELATIN IN DOGS (DATA FROM DOG 3 OBTAINED ON TENTH WEEK OF HEMORRHAGE AND INFUSION STUDIES; FOR FURTHER CONDITIONS OF EXPERIMENT, SEE TEXT)

TIME (HR.)	PLASMA PROTEIN NITROGEN (MG. %)	PLASMA GELATIN NITROGEN (MG. %)	SEDIMENTATION RATE (MM/HR.)
Before hemorrhage	840	0	1.0
Blood withdrawn (658 ml. or 3.6% of body weight) and 520 ml. of 5% gelatin infused			
1/4	326	281	70.0
2	435	178	56.0
4	482	139	50.0
7	541	110	37.0
11	566	90	30.0
16	621	65	27.0
22	701	35	28.0
30	730	32	27.0
48	774	18	43.0
168	797	12	1.0

The sedimentation rate of erythrocytes was greatly accelerated by the infused gelatin. In most instances the sedimentation rate decreased approximately at the same rate as the gelatin concentration in the plasma decreased. In all instances, the rate had returned to normal the following week (Table III).

Plasma gelatin determinations revealed the presence of approximately 80 per cent of that infused at one-fourth hour after infusion, 50 per cent at four hours, and 25 per cent at sixteen hours. Urine gelatin determinations revealed that only 37 to 62 per cent of the gelatin infused was excreted unchanged (Table IV). This occurred within twenty-four hours. Gelatin determinations on aqueous extracts of the liver and kidneys of one dog at autopsy eight days after thirteen infusions indicated that there was no free gelatin stored in these organs.

TABLE IV. RATE OF APPEARANCE OF GELATIN IN URINE FOR THREE SUCCESSIVE WEEKS  
(DATA FROM DOG 3)

FIRST WEEK		SECOND WEEK		THIRD WEEK	
PERIOD AFTER INFUSION (HR.)	AMOUNT OF GELATIN IN URINE (% OF GELATIN INFUSED)	PERIOD AFTER INFUSION (HR.)	AMOUNT OF GELATIN IN URINE (% OF GELATIN INFUSED)	PERIOD AFTER INFUSION (HR.)	AMOUNT OF GELATIN IN URINE (% OF GELATIN INFUSED)
0-2	38.5	0-6	18.8	0-6	36.7
2-16	23.0	6-18	18.3	6-18	20.3
16-40	0.0				
Total excreted	61.5		37.1		57.0

Observations at autopsy revealed no gross pathology, except for scar tissue and thrombi in some instances at the sites of bleeding and gelatin infusion as noted previously.

Histologic examination of the heart, coronary vessels, aorta, carotid arteries, jugular veins, and renal arteries showed, in some instances, changes attributable only to prior disease. There was no vacuolization or subendothelial edema of the arteries and arterioles. Sections of the trachea, lungs, stomach, intestines, spleen, gall bladder, adrenals, striated muscle, brain, meninges, and gonads likewise showed no changes attributable to gelatin.

The parenchyma of the liver was normal. Many of the Kupffer cells, however, in all gelatin-infused dogs contained sudanophile droplets. Those in the areas of the acini around the periportal fields were most affected. The Kupffer cells in nine of twenty-four control sections of liver tissue showed moderate fat staining. The Kupffer cells of human liver, according to Levine,<sup>28</sup> normally contain neutral fat. The Kupffer cells of one of the dogs from which a portion of the liver was removed prior to gelatin infusions were fat-free. Immediately after completion of the series of infusions, the Kupffer cells were loaded with sudanophile droplets. At autopsy, seven weeks later, the same condition obtained. The nuclei of the Kupffer cells appeared normal in all cases.

Histologic examination of the kidneys of gelatin-infused dogs showed, in some instances, slight departures from the normal. The epithelial cells of the tubules of the proximal convolutions were sometimes swollen and occasionally vacuolated. Since gelatin is cleared through the kidneys, it is conceivable that in some animals it may have been responsible for the slight pathology observed.

## CONCLUSIONS

A highly purified and highly degraded 5 per cent gelatin solution prepared especially for intravenous therapy in human subjects was well tolerated by dogs when given repeatedly for several weeks in amounts commensurate with the fluid lost by hemorrhage. The only morphological pathology which might be attributable to gelatin in this series of dogs was the presence or increase of sudanophile droplets in the Kupffer cells. The significance of such a change is not known. It appears that the function of the Kupffer cells was not materially altered. None of our dogs developed infection of the respiratory tract or at the sites of bleeding and infusion.

The vascular pathology described by Hueper<sup>6</sup> was not observed in our series. Although Morehead and Little<sup>21</sup> found degenerative and reparative changes in the vascular system of their gelatin-infused dogs, they concluded that the lesions were not due to gelatin since similar changes were present in their control series.

Of the blood constants determined, only the sedimentation rate was altered by gelatin. This phenomenon is common to all macromolecular substances and occurs also when plasma protein concentrations are elevated. Gelatin did not interfere with the formation of hemoglobin or plasma protein, both of which were partially depleted at each hemorrhage, when infused weekly for periods as long as three months.

Gelatin appears not to be stored in the liver or kidneys. A major portion is excreted by the latter. The fate of the balance is unknown.

## SUMMARY

1. Gelatin solutions prepared for intravenous therapy proved innocuous to dogs when administered intravenously following repeated massive hemorrhages over a period of several weeks.

2. Vascular pathology was absent. The presence or increase of sudanophile droplets in the Kupffer cells was the only morphologic change attributable to gelatin.

3. Gelatin did not interfere with the formation of hemoglobin or plasma protein and appeared not to be stored in the liver or kidneys. A major portion was excreted by the kidneys.

## REFERENCES

1. Hogan, J. J.: The Intravenous Use of Colloidal (Gelatin) Solutions in Shock, *J. A. M. A.* 64: 721, 1915.
2. Wolfson, W. L., and Teller, F.: The Intravenous Use of Gelatin Solution in Hemorrhage, *Am. J. M. Sc.* 178: 562-568, 1929.
3. Taylor, N. B., and Waters, E. T.: Isinglass as a Transfusion Fluid in Hemorrhage, *Canad. Med. Assoc. J.* 44: 547, 1941.
4. Waters, E. T.: A Comparison of Isinglass and Gelatin as Blood Substitutes, *Canad. M. A. J.* 45: 395-398, 1941.
5. Gordon, H., Hoge, L. J., and Lawson, H.: Gelatin as a Substitute for Blood After Experimental Hemorrhage, *Am. J. M. Sc.* 204: 4-11, 1942.
6. Hueper, W. C.: Experimental Studies in Cardiovascular Pathology; Effects of Intravenous Injections of Solutions of Gum Arabic, Egg Albumin and Gelatin Upon the Blood and Organs of Dogs and Rabbits, *Am. J. Path.* 18: 895-933, 1942.

7. Ely, J. O., and Angulo, A. W.: Experimental Burns; Influence of Gelatin Glucose Salts Solution on Hemococoncentration of Burns, *J. Franklin Inst.* 235: 197-201, 1943
8. Grodus, F. S.: Gelatin as a Blood Substitute in Shock Due to Limb Trauma, *Federation Proc* 2: 17, 1943.
9. Ivy, A. C., Greengard, H., Stein, P. F., Jr., Grodus, F. S., and Dutton, D. F.: The Effect of Various Blood Substitutes in Resuscitation After an Otherwise Fatal Hemorrhage, *Surg., Gynec. & Obst.* 76: 85-90, 1943.
10. Janota, M., Necheles, H., Weston, R. L., Weissman, V., and Levinson, S. O.: Gelatin Infusion in Hemorrhage Shock, *Exper. Med. & Surg.* 1: 298-303, 1943.
11. Kleinberg, W., Remington, J. W., Liverole, W. J., Overman, R. R., and Swingle, W. W.: The Effectiveness of Plasma, Gelatin and Saline Transfusion in Preventing Shock Induced by Leg Muscle Trauma and Tourniquets, *Am. J. Physiol.* 140: 197-204, 1943.
12. Lawson, H., and Rehm, W. S.: The Relative Value of Various Fluids in Replenishment of Blood Lost by Hemorrhage, With Special Reference to the Value of Gelatin Solutions, *Am. J. Physiol.* 140: 131-138, 1943.
13. Little, J. M., and Wells, H. S.: Capillary Permeability to Intravenously Administered Gelatin, *Am. J. Physiol.* 138: 195-198, 1943.
14. Parkins, W. M., Koop, C. E., Riegel, C., Vars, H. M., and Lockwood, J. S.: Gelatin as a Plasma Substitute; With Particular Reference to Experimental Hemorrhage and Burn Shock, *Ann. Surg.* 118: 193-214, 1943
15. Jacobson, S. D., and Smyth, C. J.: Gelatin as a Substitute for Plasma, *Arch. Int. Med.* 74: 251-257, 1944
16. Kozoll, D. D., Popper, H., Steigmann, F., and Volk, B. W.: Use of Gelatin Solution in the Treatment of Human Shock, *Am. J. M. Sc.* 208: 111-117, 1944.
17. Robschewitz Robbins, F. W., Miller, L. L., and Whipple, G. H.: Gelatin—Its Usefulness and Toxicity, *J. Exper. Med.* 80: 115-164, 1944.
18. Swingle, W. W., Kleinberg, W., and Hays, H. W.: A Study of Gelatin and Saline as Plasma Substitutes, *Am. J. Physiol.* 141: 329-337, 1944.
19. Steigmann, F., Meyer, K., Kozoll, D. D., Volk, B. W., and Popper, H.: Gelatin Solution as a Plasma Substitute, *Am. J. Clin. Path.* 15: 223-227, 1945.
20. Hoffman, W. S., and Kozoll, D. D.: The Fate of Intravenously Injected Gelatin in Human Subjects, *J. Clin. Investigation* 25: 375-385, 1946.
21. Morehead, R. P., and Little, J. M.: A Morphological Study Following the Intravenous Administration of Gelatin Solutions to Dogs, *Am. J. Path.* 21: 333-338, 1945.
22. Westergren, A.: Studies of the Suspension Stability of the Blood in Pulmonary Tuberculosis, *Acta Med. Scandinav.* 54: 247, 1921.
23. Levine, V.: Normal Fat Content of Kupffer Cells; Histologic Study, *Arch. Path.* 14: 345-352, 1932.
24. Patek, A. J., Kendall, F. E., Victor, J., Lowell, A., Coleher, H., and Seegal, D.: Venous Thrombosis After Infusion With Gelatin Solutions Containing Mercurial Preservatives, *Am. J. M. Sc.* 212: 561-564, 1946.



## ATTEMPTS AT THE EXPERIMENTAL PRODUCTION OF ARTHRITIS

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A RELATIONSHIP of the endocrine system to the phenomena of arthritis has long been suspected. Even before the development of modern endocrinology, it was recognized that the menopause seemed to have a significant influence upon the development of hypertrophic or osteoarthritis, and the term arthritis of the menopause appears in the literature as far back as 1855.

Recently an attempt has been made by one of us (R. P.), in conjunction with Scull, to integrate known and suspected factors within the neuroendocrine field in the pathogenesis of rheumatic disorders.<sup>1</sup>

Efforts have been made to simulate in experimental animals the phenomena of arthritis in some of its manifestations by means of injection of hormonal material, by the ablation of certain hormone-yielding organs, and by other steps of the kind. Among the most significant studies in this connection have been those of the Silberbergs<sup>2</sup> who have succeeded in producing some of the pathologic picture of hypertrophic or osteoarthritis in experimental animals by injection of extracts of the pituitary. Another attempt of the same general nature has been made by Selye,<sup>3</sup> who describes the induction of a number of phenomena characteristic of the arthritic state as the result of the injection of desoxycorticosterone acetate with the exhibition of certain other concomitant influences.

Selye<sup>3</sup> reports that the administration of desoxycorticosterone acetate in comparatively large doses causes the formation of "Aschoff bodies in the heart and the presence of periarteritis nodosa, a condition occasionally seen in man following an attack of rheumatic fever. Since in addition choreiform twitches and a few rare cases of arthritis were encountered in desoxycorticosterone acetate treated animals, it may be said that all the elements of the complex rheumatic syndrome were reproduced in experimental animals."

He further states, "The arthritic lesions, which are rarely seen in the intact desoxycorticosterone acetate treated rat, develop with great frequency in similarly treated thyroidectomized or adrenalectomized animals, especially if these are kept in cold surroundings."

He also says, "Previous experiments revealed that unilateral nephrectomy and sodium chloride treatment increase the sensitivity of rats to many overdosage effects of desoxycorticosterone acetate." In his experiments "all animals were sensitized by ablation of the left kidney 5 days before injections were started and given 1% NaCl to drink beginning on the day of operation."

It is obvious that importance attaches to conclusions of this nature and it was therefore decided to repeat, so far as possible, Selye's experiments.

From the Abington Memorial Hospital, Abington, Pa.

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*Experiment 1 (Divided Into Three Groups).—*

*Group A:* Eight female albino rats weighing 20 grams each were put on Fox chow plus 1 per cent NaCl to drink, and six of them were given injections of 3 mg. desoxycorticosterone acetate\* twice daily for forty-two days. To serve as controls, two received no injections. The temperature of the animal room varied from 55 to 80° F. Three of the six injected rats showed some redness and swelling of the hind feet and knee joints eleven to eighteen days after injection. This subsided and reoccurred from time to time, disappearing before autopsy. The two controls showed no gross lesions. At autopsy six of the eight rats, including both controls, were found to have parasitic infection of the livers, the parasite being a strobilocercus of the *Taenia Taeniaformis*, a common



Fig 1.—One articular surface of experimentally induced arthritis shows focal destruction of cartilage and underlying bone. The normal tissues are replaced by seminecrotic material, proliferating cartilage, and fibroblasts.

tapeworm of the domestic cat, the intermediate host being rats and mice. Sections were made of the hind legs and feet. The three affected rats showing gross swelling revealed microscopic joint changes. These were slight proliferation of the capsule with the lining of the cells piled up over a small ulcer in some places; loss of cartilage; a proliferation of fibroblasts replacing the ulcerated cartilage; marked focal destruction of articular cartilage on both articular surfaces; and adhesions extending from one ulcerated area to the other with loose connective tissue replacement (Fig. 1).

\*The desoxycorticosterone acetate used in these experiments was made available through the courtesy of Dr. Edward Henderson, Director, Division of Clinical Research, Schering Corporation, Bloomfield, N. J.

Of the two control rats receiving no injections, one leg joint of one rat showed one-third of the joint surface of the cartilage to be replaced by irregular masses of proliferating connective tissue.

*Group B:* A similar group of eight rats was given Fox chow and plain water to drink and was injected identically with Group A, in the same room, at the same time, for the same length of time, to determine whether the absence of 1 per cent salt made the animals more or less susceptible to arthritis. There was never any gross redness or swelling. One injected rat died. One control was not sacrificed. At autopsy, of the five injected rats and one control, three, including the control, were found to be infected with parasites. Sections of the hind legs were made and only one of the injected rats showed any joint change. In this animal one joint surface showed a necrotic area in the cartilage.

Within the joint cavity of another joint, seminecrotic tissue was observed to be attached to the synovia. The control rat was negative.

*Group C:* At the same time eight rats varying in weight from 25 to 86 grams were put on Fox chow plus 1 per cent NaCl, and four were given injections. The remainder served as controls. All were placed in a room without heat where the temperature was 40 to 55° C. The four injected rats died in a few days and four more, weighing 116 to 122 grams, were substituted. The controls which did not succumb weighed 82 to 86 grams. The experiment was discontinued after thirty-five days, since there was no gross redness or swelling. At autopsy no liver parasites were found. One control rat and the four injected rats were sectioned. One test rat showed proliferation of connective tissue from the synovia into one joint cavity.

It may be concluded from this experiment that:

1. Young rats, overdosed with desoxycorticosterone acetate and given 1 per cent NaCl to drink, showed joint changes in 50 per cent of the experimental animals, somewhat resembling certain phases of hypertrophic or osteoarthritis found in man. Some joint change of similar nature was seen in one joint of one control.

2. The subjection of experimental rats to environmental cold, as described, does not substantiate Selye's findings, since no evidence of gross swelling or crippling resulted.

3. The rats not ingesting salt showed no marked incidence of gross or microscopic changes in the joints.

*Experiment 2.*—This experiment was carried out on mononephrectomized and bilateral adrenalectomized rats. The following steps were employed in each operative procedure:

1. The rat was anesthetized with ether.
2. The rat under anesthesia was secured to an operative board of soft wood by means of rubber bands looped about the feet and over hooks which were attached to the board.
3. The operative area was clipped with scissors and then shaved.
4. The skin of the operative site was painted with tincture of metaphen.
5. Sterile draping of the operative site was performed.

6. The surgeon used sterile gloves after thoroughly scrubbing his hands with soap and water.

7. Sterile instruments were used throughout.

8. After the operation had been completed, the rat was kept in a warm cage until out of anesthesia.

The surgical technique employed in each experiment is now given in detail.

*Technique of Unilateral Nephrectomy and Bilateral Adrenalectomy:* Ether anesthesia was used and the rats were placed in a prone position. A dorsal midline incision was made through the skin at the level of the kidneys. The subcutaneous fascia was incised on the right, exposing the muscle. A longitudinal incision was then made along the lateral border of the right dorsal muscle mass. The muscle was retracted medially and the kidney and adrenal exposed. The right kidney and adrenal were delivered. The kidney pedicle was ligated with No. 00 plain catgut and the kidney and adrenal excised. Similar exposure was then made on the left side. The kidney was delivered and the adrenal was displaced upward, detached from the upper pole of the kidney, and removed. The kidney was returned to its anatomic position. The skin was then closed with interrupted fine silk sutures.

Thirteen female albino rats weighing from 40 to 50 grams were mononephrectomized, and seven of these were bilaterally adrenalectomized. They were given 1 per cent NaCl to drink and Fox chow. Five days later, weighing 65 to 90 grams, they were given injections of 3 mg. of desoxycorticosterone acetate twice daily for twenty-eight days. This was given to the seven adrenalectomized, mononephrectomized rats and to four of the six mononephrectomized rats. The mononephrectomized and one intact rat were kept as controls, receiving no injections. They were all kept in a warm room.

No redness or gross swelling was observed. At autopsy none of the rats showed parasitic infestation. The hind legs were examined microscopically with the following results:

1. Two of the seven adrenalectomized, mononephrectomized rats showed some joint changes.

2. One of the four mononephrectomized rats developed a joint showing practically complete obliteration of the joint cavity due to adhesions of adult connective tissue between both articular surfaces. The same joint showed destruction, and connective tissue replacement, of articular cartilage.

3. One control rat, nephrectomized, showed slight absorption of cartilage and slight proliferation of endothelial cells at the attachment of the synovia.

It is evident from these experiments that the operations discussed did not contribute further to the development of arthritis. Normal rats overdosed with desoxycorticosterone acetate showed more evidence of joint changes than did those operated upon.

*Experiment 3.*—Eight rats were mononephrectomized and thyroidectomized as follows:

The previously described technique for unilateral nephrectomy was observed. The animal was then placed in the dorsal position for *thyroidectomy*. The head of the animal was kept in extension by means of a rubber band looped over the upper incisors. A ventral midline incision was made in the neck. The salivary glands were freed at the midline and retracted laterally. The sternohyoid muscle was divided in the midline. The thyroid was exposed by careful, blunt dissection with a small curved hemostat. The gland was grasped with

tooth forceps, and while retraction was exerted upward and laterally the gland was dissected free from its attachments by means of a needle. Bleeding was negligible when the gland was removed by this technique. The skin was then closed with interrupted fine silk.

Five days after operation, the eight rats were injected with 3 mg. of desoxycorticosterone acetate twice daily for a total of ninety injections.

None of these rats showed any gross redness or swelling. One died at five weeks. Since these rats showed no gross evidence of arthritis, it was decided to add one more stress in the form of an injection of bacterin or bacterial toxin. This, when given to human beings, often causes a systemic reaction such as chills and fever. With this thought in mind, it was decided to subject these operated rats, overdosed with desoxycorticosterone acetate, to increasing doses of whole typhoid bacterin and serobacterin.

The remaining seven rats were divided into three groups:

Two rats were given .3 ml. of typhoid whole bacterin.

Three rats were given .3 ml. of serobacterin typhoid.

Two rats were not given any bacterin, being left as controls.

Two normal rats were used as controls on the bacterin alone, one receiving the serobacterin, the other, the whole bacterin. The doses of bacterin were given at five-day intervals and increased to 1 ml., the schedule of dosage being .3, .6, .8, 1 ml., and 1 ml. Since these injections did not seem to have any gross effect on the health of the rats, all were sacrificed and autopsied.

The two control rats, receiving typhoid bacterin *only*, showed no joint changes.

Of the three remaining rats, only one of which had received typhoid bacterin, all showed extensive ulcerations and connective tissue replacement of articular cartilage by fairly adult connective tissue.

The two control rats, receiving typhoid bacterin *only*, showed no joint changes.

This experiment with mononephrectomized, thyroidectomized rats subjected to overdosage of desoxycorticosterone acetate and the addition of typhoid bacterin did not increase the extent or incidence of joint changes.

*Experiment 4.*—Another group of fifteen rats weighing 30 grams was treated as follows:

Seven were mononephrectomized as previously described, and ovariectomized.

Four were ovariectomized. In one the testicles were removed.

*Technique of Bilateral Oophorectomy:* Ether anesthesia was used and the rats were placed in a prone position. The right ovary was exposed through the thin muscle wall just below the dorsal muscle mass. Hemostasis was then insured by ligation of the upper horn of the uterus on the right side with No. 00 plain catgut. The right ovary and the oviduct were then removed. A similar technique was used to remove the left ovary and oviduct. The skin incision was closed with interrupted fine silk sutures.

Three of the fifteen rats were left intact as controls. All were put on Fox chow and 1 per cent NaCl. Five days after operation all were given 3 mg. of desoxycorticosterone acetate twice daily for thirty days, a total of sixty injections.

The rats gained weight and showed no gross evidence of arthritis. All were sacrificed and autopsied. There was no parasitic infection of the livers.

One rat which had a stitch abscess for twenty days before the infected stitch was removed and the lesion healed was watched especially to see if it would show any joint involvement due to the added stress of infection; it did not, however, and microscopic findings of its joints were negative.

The results of this experiment were:

Three of the seven mononephrectomized, gonectomized rats had some minor changes of the cartilage and underlying bone of the leg joints.

Two of the five ovariectomized rats had a few small foci of erosion of articular cartilage extending down to the bone.

Two of the three control rats, having no operations, showed focal ulcerations or erosion of cartilage in some places extending down to the bone.

This experiment reveals that the operative procedures of mononephrectomy and ovariectomy, or ovariectomy alone, do not lead to joint changes resembling "rheumatic and rheumatoid conditions in man" with a frequency greater than in normal rats which are unoperated and overdosed with desoxycorticosterone acetate.

Since the controls in these experiments showed some joint change, a food experiment was conducted.

*Experiment 5.*—Twenty male and twenty female rats, most of them weighing 30 to 50 grams, a few weighing up to 100 grams, were divided into four groups, five of each sex of varying weight in each group. Both sexes were included to ascertain if one sex were more prone to arthritis than the other.

Group 1 was given Fox chow plus 1 per cent of NaCl.

Group 2 was given Fox chow plus 2 per cent of NaCl.

Group 3 was given Fox chow plus plain water.

Group 4 was given no Fox chow, but bread, greens such as lettuce or celery, and water.

All groups were kept on schedule during the summer months, from June 15 to September 26, when they were sacrificed with the following results:

1. None of the rats at any time showed any gross evidence of arthritis.
2. All rats of the group on 2 per cent NaCl were stunted in growth, scaly, dirty, and emaciated. They gained weight, but slowly.
3. Twenty-three of the thirty rats eating Fox chow showed liver parasites.
4. None of the rats, male or female, of Group 1 or 2 showed any microscopic joint changes.
5. Only three of Group 3 showed any microscopic joint change, this being found in female rats only. This joint change is described as fairly extensive destruction of articular cartilage with adhesions between articular surfaces.
6. Group 4, however (not on Fox chow), showed no liver parasites, was grossly healthy, and gained weight at a normal rate, but microscopically six of seven presented joint changes.

These joint changes constituted the most marked microscopic findings of the entire group of experiments undertaken.

The changes consisted of a fairly extensive destruction of articular cartilage, ulceration, and adhesions connecting the ulcerating surfaces (Fig. 2).

Finally, reviewing all the variations carried out in these five experiments and the results obtained, the work was concluded by repeating the first experiment.



Fig. 2—One articular cartilage, in experimentally induced arthritis, shows a superficial ulcer in which the cartilage cells have been replaced by seminecrotic material.

*Experiment 6.*—Rats of our own raising, weighing 30 grams, were used. Several were sacrificed before beginning the experiment to see if they were free of liver parasites. The Fox chow was sterilized, as were all the cages, to eliminate, if possible, parasitic infestation.

It was decided to use two preparations of desoxycorticosterone acetate; a 3 mg. emulsion of crystalline desoxycorticosterone, and a 5 mg. in 1 ml. of peanut oil.

Sixteen rats weighing 30 grams were used. Eight were given 3 mg. of the emulsion twice daily. Two were kept as controls with no injections. Six were given .5 ml. intramuscularly twice daily of the oil preparation one dose in the muscle of each hind leg. All were given sterilized Fox chow and 1 per cent NaCl for drinking water. The eight rats received injections for a period of thirty-nine days, at which time injections were discontinued.

There was no gross evidence of arthritis in either test or control rats, and all were sacrificed twenty-seven days later. The six rats on oil preparation showed much edema of the hind legs and feet which was believed to be unabsorbed oil.

The rats received a total of 18 ml. per rat over a period of twenty-four days. The day after the last injection one of these rats was sacrificed and

about 7 ml. of oil were aspirated from the tissue of the hind legs and the peritoneal cavity. The remaining animals were kept on their food, plus 1 per cent salt solution for thirty-three days longer to allow for further absorption of oil and subsidence of edema.

At autopsy no liver parasites were found. Eight of the sixteen rats, one of which was a control, or 50 per cent, showed focal destruction of cartilage and ulceration of cartilage with loose connective tissue stretching across the joint cavity from ulcer to ulcer. Some of the underlying bone was seminecrotic in the oil-treated rats (Fig. 3). These joint changes were most marked in the oil-treated animals.

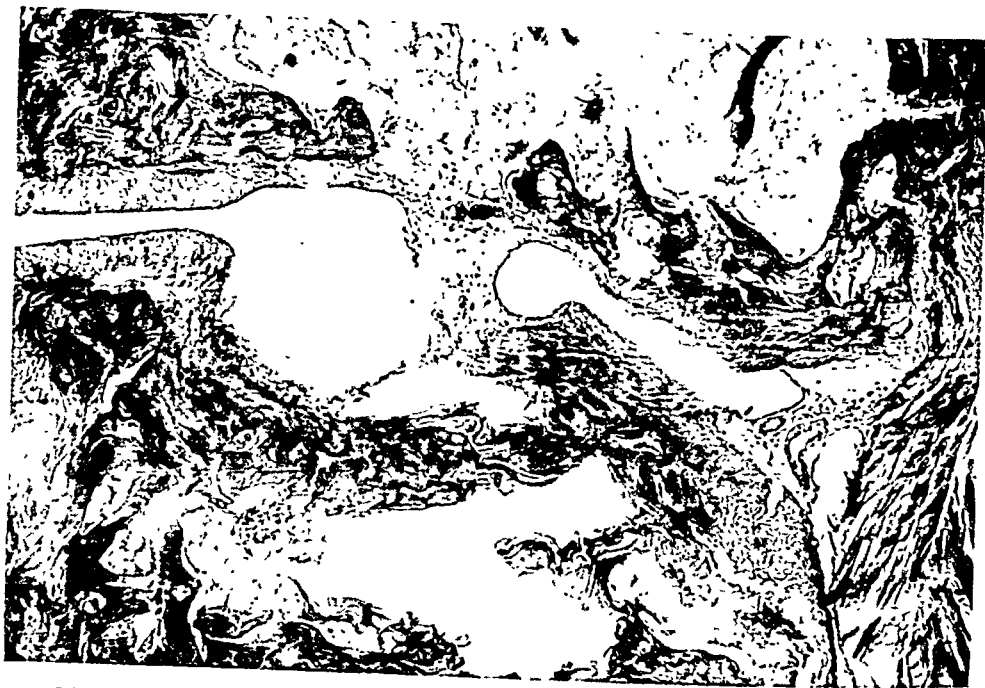


Fig. 3.—Focal destruction or ulceration of cartilage and underlying bone on opposing articular surfaces in experimentally induced arthritis. The ulcerated areas are joined by a band or adhesion of young connective tissue which stretches across the joint cavity.

#### COMMENT

It is accepted that so-called spontaneous arthritis occurs sometimes in experimental animals for reasons that are not clear. In an attempt to meet this situation efforts were made to eliminate the influence of parasitic infection which was encountered in Experiments 1 and 2. By the same token, dietetic experiments were conducted to show that experimental rats on a balanced diet do not show the arthritic changes encountered in the rats fed an unbalanced diet. Evidence has been adduced that there may be an apparently spontaneous occurrence of arthritis in rats due to a "pleuro-pneumonia-like" organism.<sup>4</sup> Normal animals, however, may also harbor this organism. The animals, in the series here reported, that showed arthritis gave no clinical evidence of systemic illness and autopsy revealed no pneumonitis or other evidence of pulmonary disease.



It is further to be observed that as far as so-called spontaneous arthritis is concerned, a chief conclusion of this work has to do with failure to reproduce arthritis as the result of certain operative procedures followed by administration of desoxycorticosterone acetate.

#### SUMMARY

Arthritic-like lesions were encountered in groups of rats maintained on standard laboratory rations and on grossly unbalanced diets and in animals receiving injections of massive doses of desoxycorticosterone acetate. In one group receiving massive doses of desoxycorticosterone acetate, together with a one per cent NaCl solution for drinking water, joint changes were formed in 50 per cent of young female white rats. These results are at variance with the findings of certain other workers that arthritic lesions are rarely seen in intact desoxycorticosterone-treated rats. Attempts to increase the incidence and severity of these arthritic-like lesions by exposure to cold, by thyroidectomy, adrenalectomy and gonadectomy were unsuccessful. The changes observed in the joints are those of focal areas of degeneration and ulceration in the articular cartilages.

#### REFERENCES

1. Pemberton, Ralph, and Scull, C. W.: The Role of Central Factors in the Pathogenesis of Rheumatic Disorders, *Ann. Int. Med.* 19: 482, 1943.
2. Silberberg, M., and Silberberg, R.: Combined Effects of Estrogen and Anterior Hypophyseal Extract on the Skeleton of Growing Mice, *Arch. Path.* 39: 381-387, 1945.
3. Selye, Hans, Jr.: Hormonal Production of Arthritis, *J. A. M. A.* 124: 201, 1944.
4. Jordan, E. O., and Burrows, William: *Textbook of Bacteriology*, Philadelphia, 1943, W. B. Saunders Company, p. 672.

## STUDIES ON THE NEPHROTOXIC ACTION OF *DL*-SERINE IN THE RAT

### III. THE INFLUENCE OF EXPERIMENTAL HYDRONEPHROSIS

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EXTENSIVE renal necrosis in the kidneys of young male rats has been shown to be caused by *DL*-serine ( $\alpha$ -amino- $\beta$ -hydroxy propionic acid).<sup>1,2</sup> The protective action of various amino acids and related compounds against the damaging action of *DL*-serine<sup>1,3</sup> was reported in a previous communication. *DL*-methionine and glutathione exert a considerable protective influence. This is probably not due to SH-groups since cysteine and thioglycolic acid have only small beneficial effect and 2,3-dithiopropanol (BAL) has none. *DL*-alanine, glycine, *DL*-threonine, glycolic acid, butyric acid, and pyruvic acid have a marked protective influence. 1 (+) histidine monohydrochloride and lactic acid afford appreciable protection, and 1 (+) arginine monohydrochloride and *DL*-valine, moderate protection. Some influence was seen from 1 (+) glutamic acid, while glucose, sodium acetate, and sodium chloride were without effect. It is postulated that the protective action of these substances is due to their competitive suppression of tubular reabsorption of the injurious *DL*-serine. The present report deals with the nephrotoxic effect of *DL*-serine in the presence of hydronephrosis of one kidney.

#### METHOD

Sixteen adult male albino rats from the Wistar strain were used. Their weight varied between 195 and 360 grams. Adult male animals were previously found to be as susceptible as young rats to the nephrotoxic action of *DL*-serine.<sup>4</sup> The left ureter was ligated under sodium amytal anesthesia. *DL*-serine was injected 1, 3, 4, 6, 7, 8, 9, 12, 24, 40, 64, 88, 112, and 136 hours after ligation of the ureter. One hundred milligrams of *DL*-serine dissolved in 3 ml. of distilled water were given for each 100 grams of weight by intraperitoneal injection. The animals were sacrificed twenty-four hours later. Tissues were fixed in formalin and stained with hematoxylin-eosin. Alkaline phosphatase activity was demonstrated with Gomori's method<sup>5</sup> as modified by Kabat and Furth<sup>6</sup> in tissues fixed in cold acetone. The incubation time in the substrate mixture was two hours. No counterstain was used.

#### RESULTS

In all animals the kidneys with uninterrupted urinary flow showed extensive necrosis in the region of the cortico-medullary junction. On gross examination a small, irregularly shaped strip of grayish discoloration at the innermost portion of the cortex indicated the necrotic area (Fig. 1). The epithelium of many tubules in this area was almost completely destroyed (Fig. 2). With

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the help of vital staining with lithium carmine and by the maceration and dissection technique of Oliver,<sup>9</sup> the kidney damage was localized to the terminal segments of the proximal convoluted tubules.<sup>6</sup> There was no significant difference in the extent of renal necrosis in the obstructed as compared with the unobstructed kidney when *dl*-serine was given within three hours after the ligation of the ureter. In the kidneys that had been obstructed from four to seven hours, the extent of the necrosis was considerably decreased. In one of the experiments, only few necrotic tubules were seen in the hydronephrotic kidney of a rat injected with *dl*-serine six hours following the ligation of the ureter (Fig. 3). In all animals in which *dl*-serine was given eight or more hours



Fig. 1.—The kidneys of a male rat weighing 210 grams which was given 210 mg. *dl*-serine by intraperitoneal injection six hours after ligation of the left ureter. The animal was sacrificed twenty-four hours later. The unobstructed right kidney (top) shows an irregularly shaped strip of discoloration at the cortico-medullary junction, indicating the necrotic area. The left kidney (bottom) shows hydronephrotic dilatation without the necrotic area seen in the other kidney. Approximate magnification,  $\times 2$ .

following the obstruction of the ureter, a similar degree of almost complete protection in the obstructed kidney took place. Only very occasional tubules were necrotic. The kidneys in which the ureter had been ligated showed the typical gross and microscopic changes of experimental hydronephrosis increasing in intensity with the duration of the experiment.<sup>10</sup>

Phosphatase activity was normal in animals which were killed within thirty-six hours after the ureter had been ligated. Considerable decrease in phosphatase occurred in the remaining animals in which the obstruction had



Fig. 2.—Microscopic section of the right unobstructed kidney pictured in Fig. 1. Extensive necrosis involving the distal portion of the proximal convoluted tubules is seen. Hematoxylin-eosin  $\times 150$ .

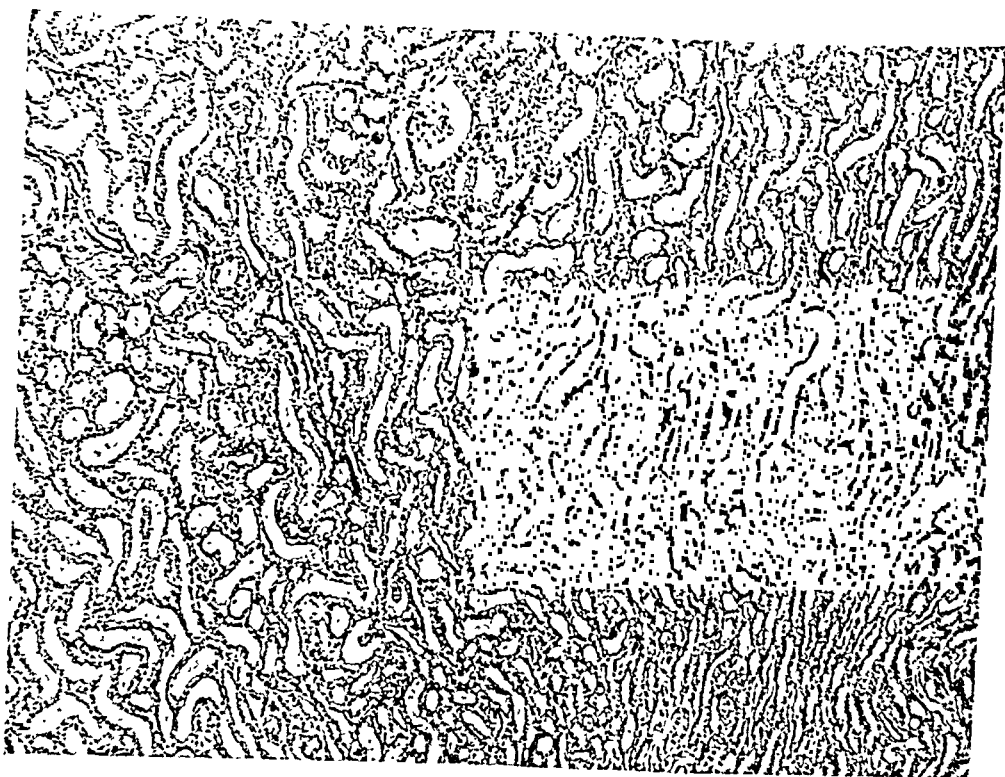


Fig. 3.—Microscopic section of the left obstructed kidney pictured in Fig. 1. Fairly generalized dilatation of the cortical tubules is seen. Renal necrosis is almost completely absent. Hematoxylin  $\times 150$ .

lasted for a longer time. The necrotic cells in the damaged proximal convoluted tubules of the unobstructed kidney revealed phosphatase activity as previously described.<sup>6</sup>

#### COMMENT

Depression of the nephrotoxic action of mercury bichloride as early as fourteen to twenty-four hours following ligation of the ureter was described by Elbe<sup>11</sup> in 1905. Elbe's findings were later confirmed by Kosugi.<sup>12</sup> Frola found a similar protective influence of hydronephrosis on the damaging effect of uranium nitrate.<sup>13</sup> These observations were recently extended by Willmer<sup>14</sup> who confirmed the protective effect of experimental hydronephrosis upon the nephrotoxic action of uranium nitrate and mercury bichloride. Sucrose,<sup>15</sup> however, as well as racemic tartaric acid and diethylene glycol produced the same amount of hydropic degeneration in the cortical tubules of the hydronephrotic and the normal kidney. In rabbits in which glomerulonephritis had been induced with duck-antirabbit serum, after the ureter in one kidney had been ligated, Reubi<sup>16</sup> observed nearly complete protection in the obstructed kidney. In this connection the observation of Fahr<sup>17</sup> is of interest. He found at autopsy on a 37-year-old woman severe subacute glomerulonephritic changes in one kidney, while the other kidney with hydronephrotic atrophy showed none.

As early as three hours following obstruction of one ureter there occurred appreciable diminution in toxicity, and six to seven hours following obstruction almost complete elimination of the injurious effect of *DL*-serine on the hydronephrotic kidney. The short time of obstruction necessary to modify the nephrotoxic action of *DL*-serine is more significant for this substance may cause microscopically demonstrable kidney changes within thirty minutes. These changes become extensive within the next few hours.<sup>6</sup>

No significant decrease in phosphatase activity is seen in the very early stage of hydronephrosis. Decrease, however, is noticeable in the hydronephrotic kidneys in the next few days and becomes progressively more pronounced. This is in full agreement with Willmer's findings.<sup>19</sup> Lipase activity in the proximal convoluted tubules of the hydronephrotic kidney shows a similar behavior.<sup>20</sup> While, therefore, the decrease in these enzymatic activities is apparently a comparatively late sign of disturbed cellular function, there are other evidences of very early changes.

Slight general dilatation of the tubules takes place within three to six hours following ligation of the ureter.<sup>21, 22</sup> Not only dilatation extending into the proximal convoluted tubules, but also flattening of epithelial cells and considerable parenchymatous and vacuolar degeneration in the cytoplasm occur within short time after ligation of the ureter.<sup>10, 12</sup> In acute experiments in which the ureter of one kidney is obstructed and the urine of both kidneys is simultaneously analyzed, a decreased concentration of nitrogenous constituents and electrolytes is found.<sup>23, 24</sup> The excretion of injected indigo carmine is considerably reduced as early as two to four hours following ligation of the ureter.<sup>23, 25</sup>

Amino acids pass the glomerular filter and are reabsorbed in the convoluted tubules.<sup>26</sup> In view of the fact the glomerular filtration in experimental hydro-

nephrosis<sup>27</sup> is probably undisturbed for some time, an impaired passage of amino acids through the glomeruli is not likely, although not impossible.<sup>28</sup> It is reasonable to assume that early functional disturbances in the convoluted tubules are responsible for the slowing up of the reabsorption of the toxic *dl*-serine. Apparently the *dl*-serine is not concentrated in the distal portion of the proximal convoluted tubules to such an extent as to permit it to exert its destructive action.

Convoluted tubules occasionally show typical necrosis even after several days of experimental hydronephrosis. A similar phenomenon was noticed previously with mercury bichloride and uranium nitrate.<sup>12, 14</sup> That even after several weeks of ligation of the ureter the convoluted tubules have not completely lost the ability to concentrate injected lithium carmine has been shown by Suzuki.<sup>29</sup>

#### SUMMARY

In adult male rats the ureter of one kidney was ligated, and in intervals of 1 to 136 hours following the obstruction of the ureter *dl*-serine was injected. The necrotizing nephrosis induced by *dl*-serine is appreciably reduced in kidneys in which the ureter has been ligated for three to seven hours. It is almost entirely suppressed if the injurious amino acid is given eight or more hours after the ligation of the ureter. It is assumed that early disturbance of tubular reabsorption does not permit the concentration of *dl*-serine in the sensitive cells of the proximal convoluted tubules to become sufficiently great to exert its destructive action.

#### REFERENCES

1. Fishman, W. H., and Artom, C.: Serine Injury, *J. Biol. Chem.* 145: 345, 1942.
2. Morehead, R. P., Fishman, W. H., and Artom, C.: Renal Injury in the Rat Following the Administration of Serine by Stomach Tube, *Am. J. Path.* 21: 803, 1945.
3. Morehead, R. P., Fishman, W. H., and Artom, C.: The Nephrotoxic Action of *dl*-Serine as Related to Certain Dietary Factors, *Am. J. Path.* 22: 385, 1946.
4. Wachstein, M.: Influence of *dl*-Methionine and Other Substances on the Nephrotoxic Action of *dl*-Serine, *Nature* 159: 236, 1947.
5. Wachstein, M.: Studies on the Nephrotic Action of *dl*-Serine in the Rat. II. The Influence of Various Amino Acids and Other Compounds, *Arch. Path.* 43: 515, 1947.
6. Wachstein, M.: Studies on the Nephrotoxic Action of *dl*-Serine in the Rat. I. Localization of the Kidney Damage, Phosphatase Activity and Influence of Age, Sex, Time and Dose, *Arch. Path.* 43: 503, 1947.
7. Gomori, G.: Microtechnical Demonstration of Phosphatase in Tissue Sections, *Proc. Soc. Exper. Biol. & Med.* 42: 23, 1939.
8. Kabat, E. A., and Furth, J.: A Histochemical Study of the Distribution of Alkaline Phosphatase in Various Normal and Neoplastic Tissues, *Am. J. Path.* 17: 303, 1941.
9. Oliver, J.: New Directions in Renal Morphology: a Method, Its Results and Its Future, *Harvey Lect.* 40: 102, 1944-45.
10. Strong, K. C.: Plastic Studies in Abnormal Renal Architecture. V. The Parenchymal Alterations in Experimental Hydronephrosis, *Arch. Path.* 29: 77, 1940.
11. Elbe: Die Nieren und Darmveränderungen bei der Sublimatvergiftung des Kaninchens und ihre Abhängigkeit vom Gefäßnervensystem, *Virchow's Arch. f. path. Anat.* [etc.], *Berl.* 182: 445, 1905.
12. Kosugi, T.: Beiträge zur Morphologie der Nierenfunktion, *Beitr. z. path. Anat. u. z. allg. Path.*, Jena 77: 1, 1927.
13. Frola, E.: Sulla Nefrite Tossica Sperimentale Nella Idronefrosi, *Pathologica* 21: 109, 1929.
14. Willmer, H. A.: Nephrotoxic Effect of Poisons Active on Convoluted Tubules in Presence of Hydronephrosis of One Kidney, *Arch. Path.* 38: 85, 1944.
15. Willmer, H. A.: The Mechanism of Sucrose Damage of the Kidney Tubules, *Am. J. Physiol.* 141: 431, 1944.

16. Reubi, F.: Hydronephrose et Glomerulonephrite, Schweiz. med. Wchnschr. 46: 457, 1946.
17. Fahr, T. H.: Die Funktionsbehinderung der Niere als hemmendes Moment bei der Entwicklung von Glomerulonephritis und maligner Nephrosclerose, Deutsches Arch. f. klin. Med. 191: 52, 1943.
18. Willmer, H. A.: The Disappearance of Phosphatase From the Hydronephrotic Kidney, J. Exper. Med. 78: 225, 1943.
19. Willmer, H. A.: Renal Phosphatase, Arch. Path. 37: 227, 1944.
20. Wachstein, M.: Influence of Experimental Kidney Damage on Histochemically Demonstrable Lipase Activity in the Rat. Comparison With Alkaline Phosphatase Activity, J. Exper. Med. 84: 25, 1946.
21. Fabian, E.: Die Niere des Kaninchens nach der Unterbindung ihrer Harnleiter, Biblioth. Med., Abt. Path. u. Path. Anat. 18: 1, 1904.
22. Enderlen: Experimentelle und histologische Untersuchungen über Hydronephrose und ihrer Behandlung, Verhandl. d. deutsch. Gesellsch. f. Chir., Berl. 1: 181, 1904.
23. Lindemann, W.: Ueber die Wirkung der Gegendruckerhöhung auf die Harnsecretion, Beitr. z. path. Anat. u. z. allg. Path., Jena 21: 500, 1897.
24. Pfaundler, M.: Ueber die durch Stauung im Ureter zustande kommende Veränderung der Harnsekretion, Beitr. z. chem. Phys. u. Path., Brnschw. 2: 336, 1902.
25. Kawasoye, M.: Experimentelle Studien zum künstlichen Ureterverschluss (2. Teil), Ztschr. f. gynäk. Urol., Leipz. 3: 172, 1911-12.
26. Renal Clearance of Amino Acids, Nutrition Rev. 5: 43, 1947.
27. Kitani, Y.: Hydronephrotische Atrophie oder hydronephrotische Schumpfniere? Experimentelle Untersuchungen über Hydronephrose, Virchow's Arch. f. path. Anat. [etc.], Berl. 254: 115, 1925.
28. Bell, E. T.: Renal Diseases, Philadelphia, 1946, Lea and Febiger.
29. Suzuki, T.: Zur Morphologie der Nieren Secretion unter physiologischen und pathologischen Bedingungen, Jena, 1912, Gustav Fischer.

## LABORATORY METHODS

### A METHOD FOR SHOWING ANTIBACTERIAL ACTIVITY OF MINUTE QUANTITIES OF GRAMICIDIN OR PENICILLIN

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THE test described is based on the method of Jones and Simms<sup>1</sup> who showed that bacteriostasis could be measured as a function of inhibition of colony size and hemolytic zones produced by Group A streptococci on blood agar plates.

Spingarn and King<sup>2</sup> used a similar procedure in studying the comparative susceptibility of Group A streptococci to penicillin.

By the use of this method we have been able to compare the activity of gramicidin and penicillin on a weight basis, and to determine the gramicidin content in tyrothricin samples. The following examples give an indication of the results obtained.

#### METHODS

An eighteen-hour blood broth culture of C203 (Group A) streptococcus is diluted to  $10^{-5}$  in plain broth.

Dilutions of the drug to be tested are made in a suitable diluent, that is, penicillin in water, or tyrothricin or gramicidin in 50 per cent propylene glycol-water.

Sterile defibrinated rabbit blood (taken from rabbits which had been starved for twenty-four hours prior to heart puncture) was used. We have routinely used blood which has been at least twenty-four hours old and not more than five days old.

The tests are set up as follows: To Petri dishes (100 by 15 mm.) add:

0.5 ml. defibrinated rabbit blood

0.1 ml.  $10^{-5}$  culture dilution

0.1 ml. drug dilution (do not mix with blood or culture until agar is added)

11.8 ml. proteose-tryptone agar melted and cooled to  $45^{\circ}\text{C}$ .

The contents of the plates must be thoroughly mixed to insure an even distribution of all components.

Various amounts of a drug in blood agar are made to give an adequate range of concentrations. This range should cover from 80 to 10 per cent inhibition of growth. Usually five plates are prepared to study one sample. Four of these contain different concentrations of the drug in blood agar, and one

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(containing no drug) serves as a control. This last one gives maximum hemolytic zones around each colony.

After twenty-four hours' incubation at 37°C., the diameters of the hemolytic zones are measured with an ocular micrometer and a mechanical stage micrometer. The longest diameters of the hemolytic zones around five representative colonies are measured and averaged.

*Streptococcus* C203, under the conditions employed, usually gives a hemolytic zone 2.0 mm. in diameter. Smaller colonies and hemolytic zones are indications of the activity of the drug under test. To give a figure which increases with the activity, the "inhibitory activity" is expressed as

$$100-100 \frac{(\text{mm. of inhibitory zone with drug})}{(\text{mm. of inhibitory zone with no drug})}.$$

Table I gives ranges of concentrations of gramicidin and crystalline penicillin per milliliter of blood agar which show differences that may be measured and plotted. The results shown are from duplicate assays on two samples of gramicidin and two batches of crystalline penicillin.

TABLE I. INHIBITION OF HEMOLYTIC ZONES OF *STREPTOCOCCUS* C203 IN BLOOD AGAR BY GRAMICIDIN AND PENICILLIN

DRUG	INHIBITORY ACTIVITY*				
	CONCENTRATION $\mu\text{G}$ DRUG PER ML. BLOOD AGAR				
	0.006	0.004	0.002	0.001	0.0008
Gramicidin					
15975		50*	25		5
15975	80	50	25	11.5	
23-19-B		50	25	13	8
23-19-B	70	40	20	10	7.5
Crystalline penicillin CSC					
(1)		60	40	22	18
(2)		55	35	18	

\*Inhibitory activity defined as  $100-100 \frac{(\text{mm. inhibitory zone with drug})}{(\text{mm. inhibitory zone with no drug})}$ .

It is seen in Table I that detectable inhibition can be shown by gramicidin and penicillin when present in amounts as small as 0.8 millimicrogram per milliliter of blood agar. The results appear to be reproducible as shown by the duplicate figures. On a weight basis, penicillin appears to be more active than either of the gramicidin samples.

Tyrocidine fails to inhibit the growth of *streptococcus* C203 on blood agar plates when present in a concentration of 1 microgram of blood agar. Thus the activity shown by tyrothricin samples may be referred to as being a function of the gramicidin present.

By comparing samples of tyrothricin with a standard of known gramicidin content, it is possible to judge the gramicidin content of the unknown as greater than or less than the known standard. Table II shows such comparisons.

Sample 189 (figures show triplicate measurements) was a standard containing 20 per cent gramicidin, 80 per cent tyrocidine HCl by weight.

Sample 85-15 contained 15 per cent gramicidin and 85 per cent tyrocidine HCl by weight.

The other nine samples were from lots of tyrothricin which had been shown by the Dubos<sup>3</sup> albumin broth test (streptococcus H69D) to contain more than 20 per cent gramicidin when compared with a known 20 per cent gramicidin control.

Table II shows reasonable reproducibility of the 20 per cent gramicidin control sample (189) in triplicate tests, and, as expected, considerably lower values are shown by the 15 per cent gramicidin sample (85-15).

TABLE II. INHIBITION OF HEMOLYTIC ZONES OF STREPTOCOCCUS C203 IN BLOOD AGAR BY TYROTHRIN

TYROTHRIN SAMPLE	GRAMICIDIN CONTENT (%)	INHIBITORY ACTIVITY*		
		CONCENTRATION $\mu\text{g}$ DRUG PER ML. BLOOD AGAR		
		0.01 $\mu\text{g}$	0.02 $\mu\text{g}$	0.008 $\mu\text{g}$
	20	80*	50	21.5
189	20	80	46	22
	20	80	47	20
76	?		55	25
58	?		56	30
56	?		55	28
70	?		80	60
PD	?		65	45
69	?		60	40
75	?		55	30
73	?		58	28
NP	?	70	45	21
85-15	15	60	37	5

\*Inhibitory activity defined as  $100-100 \frac{(\text{mm. inhibitory zone with drug})}{(\text{mm. inhibitory zone with no drug})}$ .

All of the other samples show a greater inhibitory value than the 20 per cent gramicidin control samples. Samples 70, PD, and 69 give particularly high values showing 60, 45, and 40 per cent inhibitory activity at the level of 0.008  $\mu\text{g}$  per milliliter of blood-agar.

It is of interest to note that an inhibitory value of approximately 50 per cent is given by 0.02 microgram per milliliter of the standard (20 per cent gramicidin, 80 per cent tyrocidine) sample (Table II) as compared with a 50 per cent inhibitory activity by 0.004 microgram per milliliter by crystalline gramicidin (Samples 15975 and 23-19-B, Table I).

#### SUMMARY

A method is described by which the inhibitory effects of millimicrogram quantities of penicillin or gramicidin can be measured.

#### REFERENCES

1. Jones, F. S., and Simms, H. S.: The Bacterial Growth Inhibitor (Lactinin) of Milk, *J. Exper. Med.* 51: 327, 1930.
2. Spingarn, C. F., and King, A. N.: Susceptibility of Serologically Typed Group A Streptococci to Penicillin, *U. S. Nav. M. Bull.* 46: 239, 1946.
3. Dubos, R. J., cited by Henderson, J.: The Status of Tyrothricin as an Antibiotic Agent for Topical Application, *J. Am. Pharm. A. (Scient. Ed.)* 35: 141, 1946.

# A STREAK PLATE METHOD FOR DETERMINING GROWTH CURVES

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WITH THE TECHNICAL ASSISTANCE OF ANNE GUNTHER

WHILE investigating the action of certain sulfur suspensions on the growth of *Staphylococcus aureus*,<sup>1</sup> a technique was developed which was far less laborious than the pipette dilution, pour plate method for demonstrating growth curves. By adherence to certain details of technique, remarkably reproducible graphs were obtained for the first eight hours of growth.

## METHOD

*Test Culture.*—The first essential is to keep the inoculum within the range of the method. A three and one-half hour culture of *Staph. aureus* (Oxford strain) in bacto nutrient broth was prepared one day before each test. Two loopfuls of a 1:20,000 dilution were spread separately on two halves of an agar plate to estimate the population. The culture was then left overnight in the icebox.

*Agar.*—Twelve to fifteen cubic centimeters of bacto nutrient agar (1.5 per cent agar) were poured into Petri plates which were stored three to four days at 37° C. to dry. This was necessary to avoid condensation on the surface of the agar which interferes with accurate bacterial counts.

*Loop.*—A standard loop of nichrome wire, which when filled held approximately 0.002 c.c. of broth, was employed. In making subcultures the tube containing the test material was held in a slightly slanted position and only the loop immersed, taking care to pick up the maximum amount of fluid. Recently Calkins<sup>2</sup> has shown that under specified conditions the quantity of fluid taken up by a loop is remarkably constant.

*Spreading a Loopful of Fluid.*—The dry agar plates were ruled horizontally with a glass pencil, the size of the area between the lines being proportional to the expected bacterial count. (When large counts are expected, a whole plate is used for one preparation.) Then one loopful from each test preparation was spread evenly over the entire surface of the agar marked out for it, care being taken to avoid cutting into the agar with the loop. It is, of course, important not to leave even tiny droplets of test fluid unspread.

*The Bacterial Count.*—The colonies were counted after eighteen to twenty-four hours' growth at 37° C. The figures obtained were multiplied by a factor of 500 to convert them to bacteria per cubic centimeter, since one loopful held 0.002 c.c. of culture. It is obvious that negative cultures might be obtained from cultures that actually contain a few organisms per cubic centimeter.

When an entire plate was used, at least 200 individual colonies (representing 100,000 organisms per cubic centimeter) were counted. When properly inoculated, none of the colonies run into one another (see Plate I).

In the first eight hours of growth, the period of interest in our present study, the number of organisms in one loop was rarely more than 200. Therefore, the graphs represent the actual number of organisms in undiluted cultures, calculated to 1 c.c. volume. When the counts were higher than 200 colonies per plate, rough estimates were made and recorded, such as 1 plus for 200 to 400 colonies; 2 plus for 400 to 600 colonies; 3 plus for 600 to 800 colonies; and 4 plus for over 800 colonies.

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The control tube was usually slightly cloudy after four hours' growth, and always definitely cloudy after six hours. In such preparations the count was always in the millions and, therefore, out of the range of this technique.

One possible source of error was that a loopful of test fluid, especially from the higher concentrations of an antibacterial agent, might have carried over sufficient material to inhibit growth. To explore this possibility, in several of our experiments one loop was spread over agar as usual, and a second loop from the same tube was mixed with melted and cooled agar for pour plates. We found that the two sets of counts were essentially alike and therefore concluded that the possibility of this error in our bacterial counts was no greater than the possibility of error with the pour plate method.

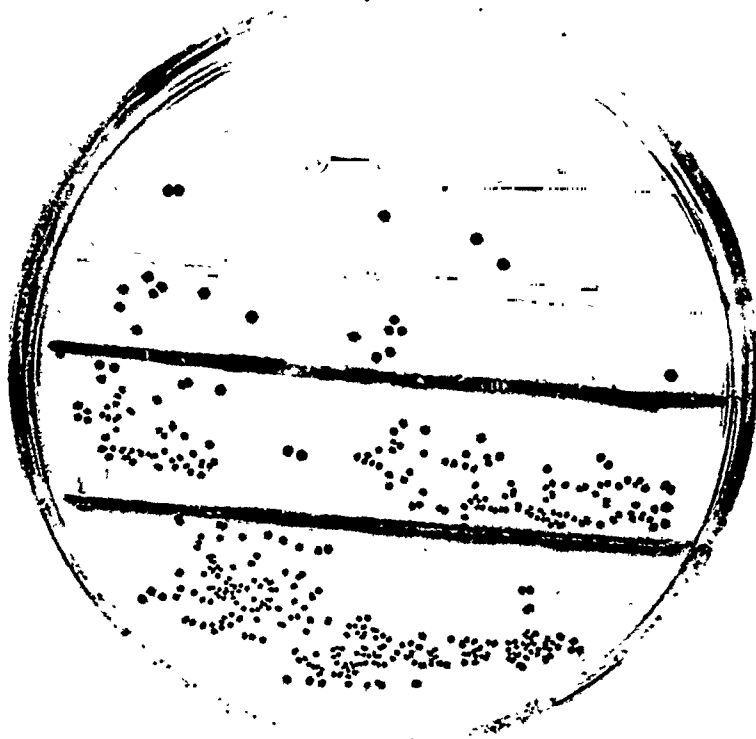


Plate I.\*—Antibacterial effect of penicillin upon the growth of *Staph. aureus*. Streak cultures of test mixture dilutions made on agar after two hours' incubation at 37° C.

READING DOWNWARD	COLONY COUNT	CALCULATED NUMBER OF ORGANISMS/C.C.
1. Penicillin content 0.1 u./c.c.	0	0
2. Penicillin content 0.075 u./c.c.	5	2,500
3. Penicillin content 0.05 u./c.c.	14	7,000
4. Penicillin content 0.025 u./c.c.	130	65,000
5. Penicillin content 0.01 u./c.c.	100	95,000

The initial number of organisms per c.c. was 27,500.

The control test after two hours' incubation at 37° C. contained over 100,000 organisms per cubic centimeter.

#### TEST PROCEDURE

The number of organisms in the test culture prepared the previous day was estimated from the preliminary plates of that day, and this culture was

\*Actual size. Photographed by Mrs. Miriam Wallace.

diluted on the day of the test so as to contain 100,000 to 600,000 cells per cubic centimeter. Appropriate dilutions of the antibacterial substance were distributed in tubes in 1 c.c. amounts, and 0.05 c.c. of the test culture was added to each tube. Two loopfuls of the control tube were at once spread out separately on two halves of an agar plate to determine the initial count. The test preparations were then placed in a water bath at 37° C. At one-hour, two-hour, or longer intervals, one loopful from each was spread over the surface of the agar allotted to it. Six antibacterial agents, each in eight dilutions, were easily tested at one-hour or two-hour intervals by one worker.

Where the antibacterial agent was dissolved in a solvent such as alcohol or carbowax,<sup>1, 3</sup> controls were made to determine whether the solvent itself affected the bacteria.

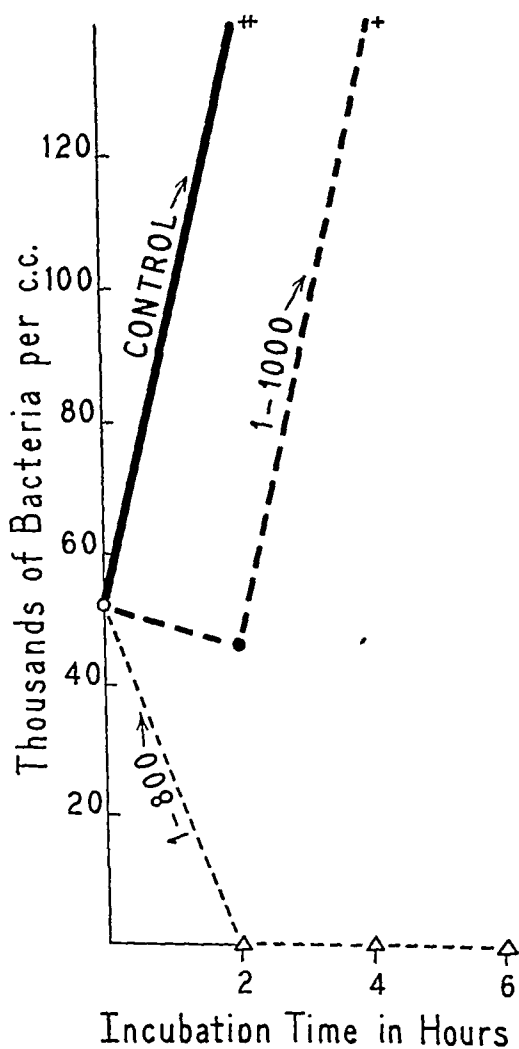


Fig. 1.—Antibacterial action of phenol for *Staph. aureus*.

## RESULTS

Figs. 1 to 11\* show the growth curves of *Staph. aureus* in the presence of the following antibacterial substances: phenol, streptothricin, streptomycin, clavacin, penicillin, protoanemonin, Manganese protoporphyrin, alcohol-sulfur, carbowax-sulfur, sulfanilamide, and pentathionic acid. Figs. 1. to 11 show single experiments, all of which have been repeated at least once with essentially similar growth curves.

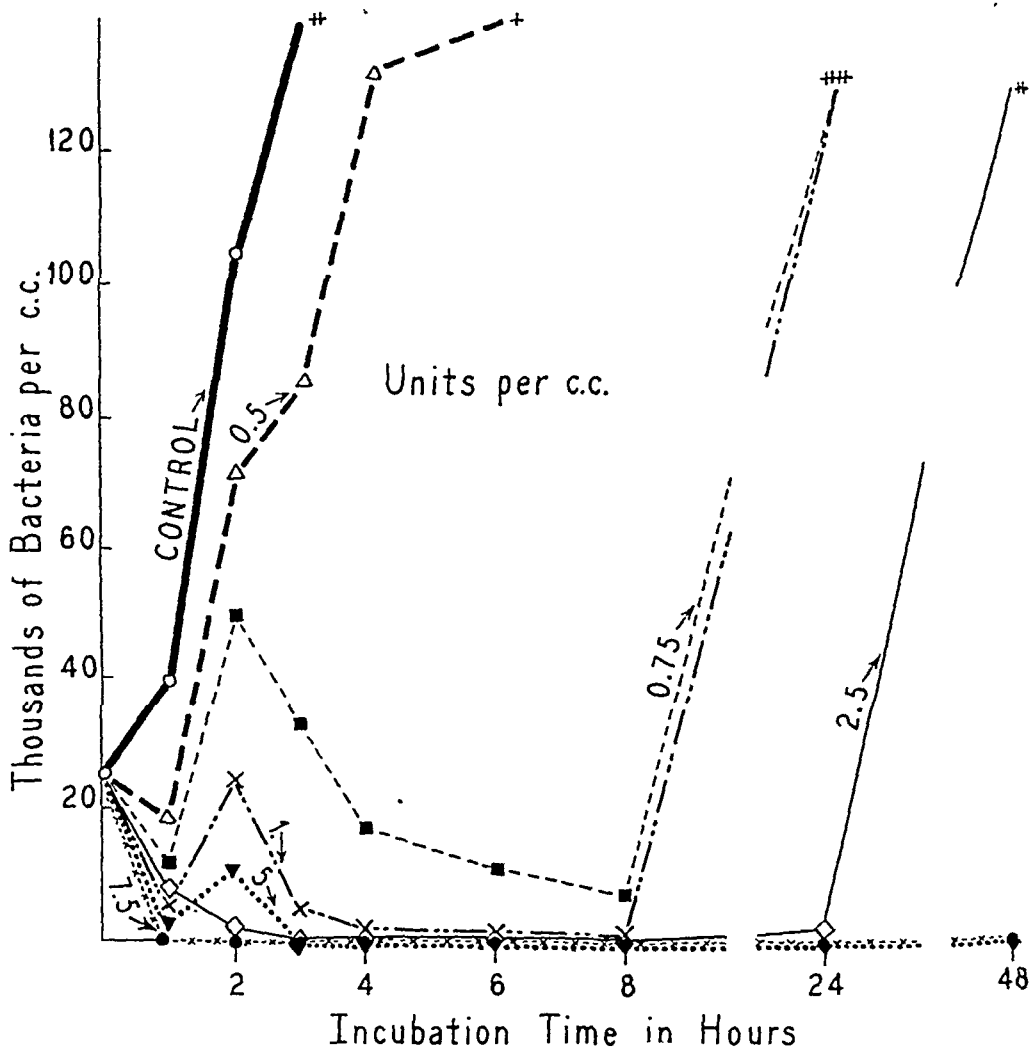


Fig. 2.—Antibacterial action of streptothricin for *Staph. aureus* (units per cubic centimeter).

Protoanemonin is an antibacterial agent, described by Seegal and Holden<sup>4</sup> and by Baer, Holden, and Seegal,<sup>5</sup> active not only against the growth of certain gram-positive and gram-negative bacteria, but also against *Cryptococcus hominis*

\*We are indebted to Miss Natalie Pearlstein for the preparation of Figs. 1 to 11.

and *Candida albicans*. The preparation used was a distillate of the stems, leaves, and blossoms of *Anemone pulsatilla*. Mn. protoporphyrin has been shown by Granick and Gilder<sup>6</sup> to inhibit a variety of organisms including *Staph. aureus* and the acid-fast bacilli in concentrations of 0.5 to 3 gamma per cubic centimeter. The sulfur preparations were suspensions of sulfur in alcohol

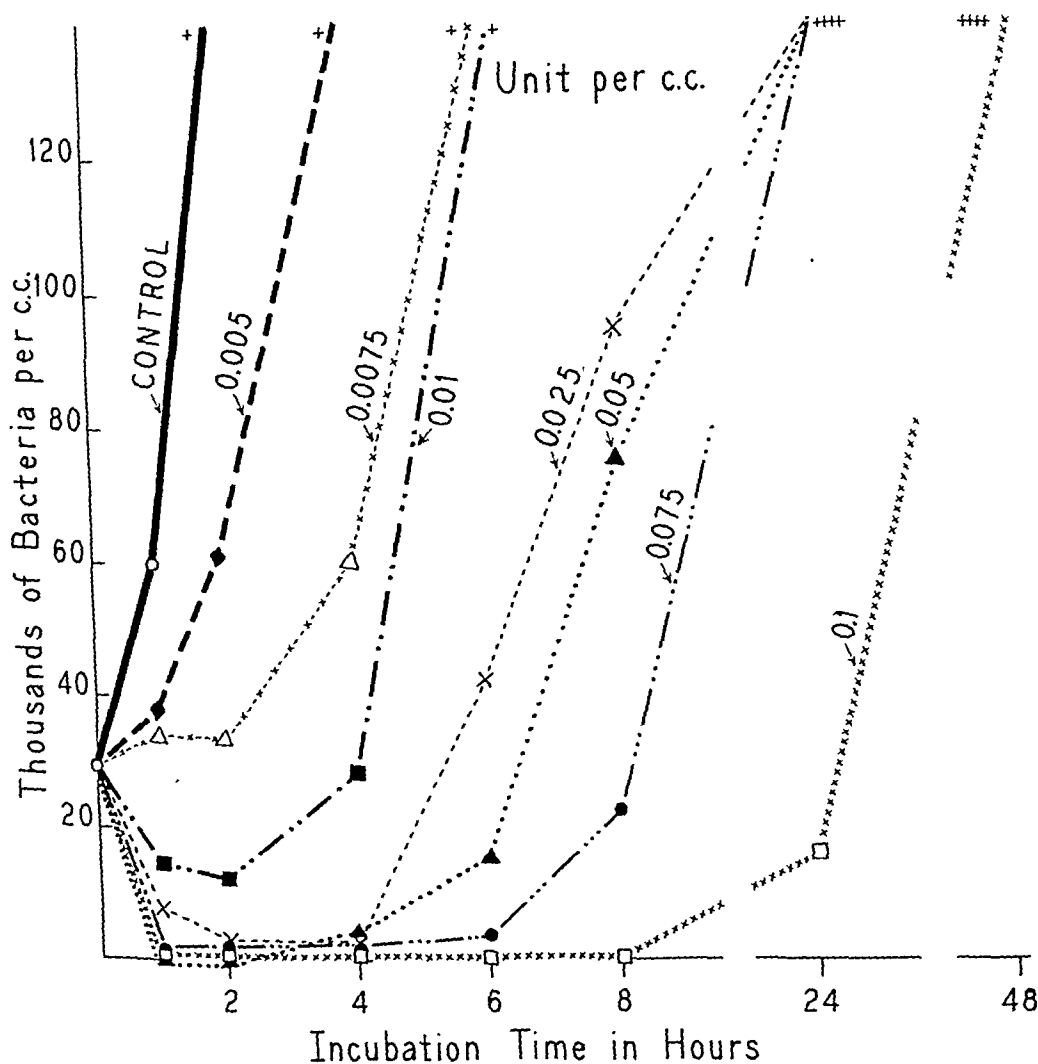


Fig. 3.—Antibacterial action of streptomycin for *Staph. aureus* (units per cubic centimeter).

(alcohol-sulfur) and in carbowax (carbowax-sulfur) which the authors have found inhibitory for *Staph. aureus* in preparations containing only 0.1 to 0.3 gamma of sulfur per cubic centimeter and for certain fungi in concentrations as low as 0.4 gamma. Pentathionic acid\* was tested because it was suspected

\*We are indebted to Dr. W. A. Knapp of the General Chemical Co., New York, N. Y., for making this material available to us. The  $H_2S_2O_8$  prepared according to the Liming method was an 8 per cent solution of  $H_2S_2O_8$  containing tetra and trithionic acids but no thiosulfates.





that if present in the sulfur preparations it might explain their extraordinary activity.

The curves showed at once important differences in the effects of these antibacterial agents in the ranges of concentration in which they were active. The more important differences are summarized in Table I. Even more interesting were the patterns of these curves obtained in the first eight hours of

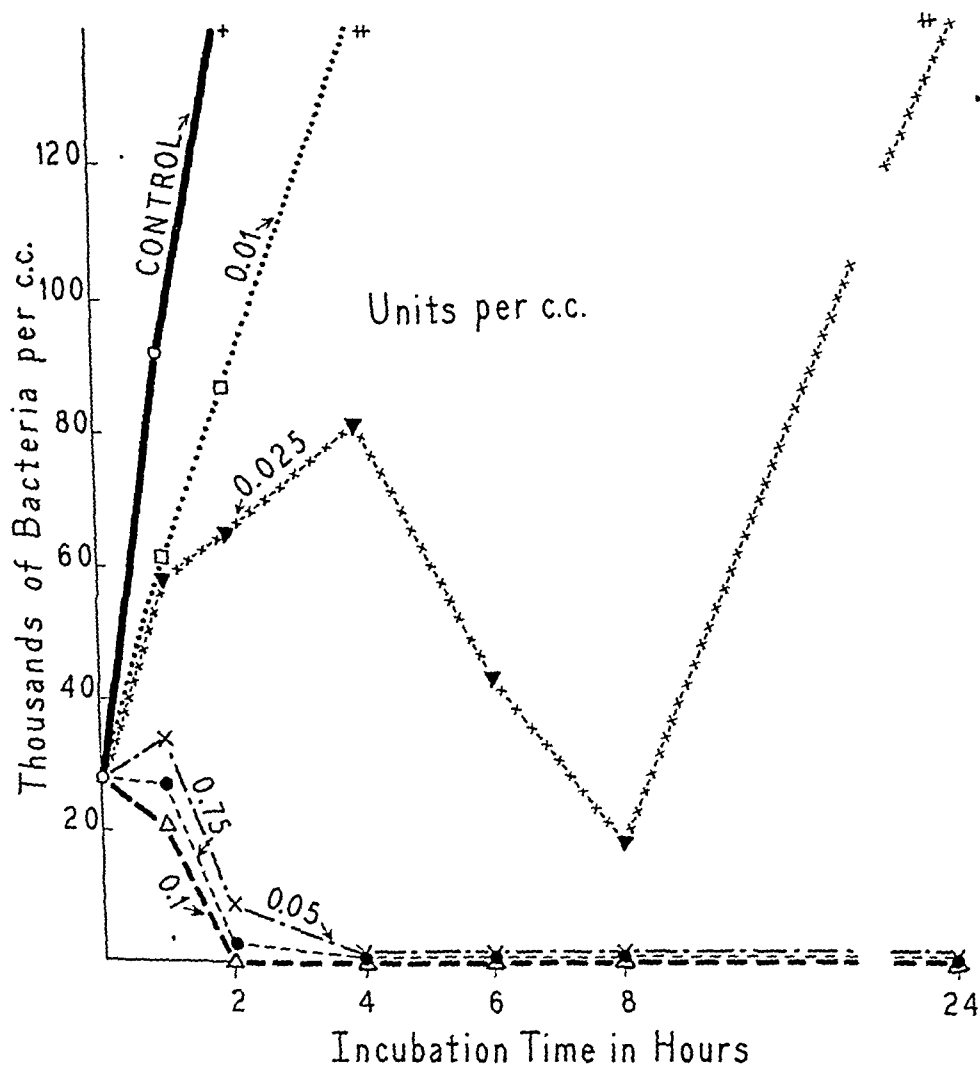


Fig. 5.—Antibacterial action of penicillin for *Staph. aureus* (units per cubic centimeter).

growth. These differed in each instance and seemed characteristic for the substances studied. The following phenomena were noted:

1. *Early Bactericidal Effect*.—The statement that certain agents were bactericidal is made with the same reservations necessary in testing for bactericidal effect by most methods in current use. It was not proved that the effect was

TABLE I. COMPARISON OF BACTERICIDAL AND BACTERIOSTATIC EFFECTS OF VARIOUS ANTIBACTERIAL AGENTS

1 ANTIBACTERIAL AGENT	2 MINIMUM BACTERICIDAL DOSE	3 BACTERICIDAL EFFECT IN HOURS	4 MAXIMUM NONINHIBIT- ING DOSE†	5 RATIO COLUMN 2 COLUMN 4
Group 1				
Phenol	1-800*	2	1-1,000	1.25
Streptothricin	5 u./c.c.*	3	0.5 u./c.c.	10
Streptomycin	0.2 u./c.c.*	1	0.005	40
Clavacin	25 µg c.c.*	6	5 µg/c.c.	5
Penicillin	0.05 u./c.c.*	4	0.01 u./c.c.	5
Protoanemonin	1-20,000*	8	1-60,000	3
Group 2	10 µg/c.c.†	—	0.125 µg/ c.c.	80
Mn. Protoporphyrin	1-100†	—	1-51,200	512
Alcohol-sulfur	1-100†	—	1-800,000	8,000
* Carbowax-sulfur				

\*No growth from one loop (0.002 c.c.) inoculum.

†No bactericidal effect in largest amounts tested in eight hours.

‡Maximum dose of antibacterial agent giving curve approximately like that of control.

Doses listed delayed growth slightly. See Fig. 2, 0.5 unit curve; Fig. 3, 0.005 unit curve; Fig. 4, 5 µg curve, etc.

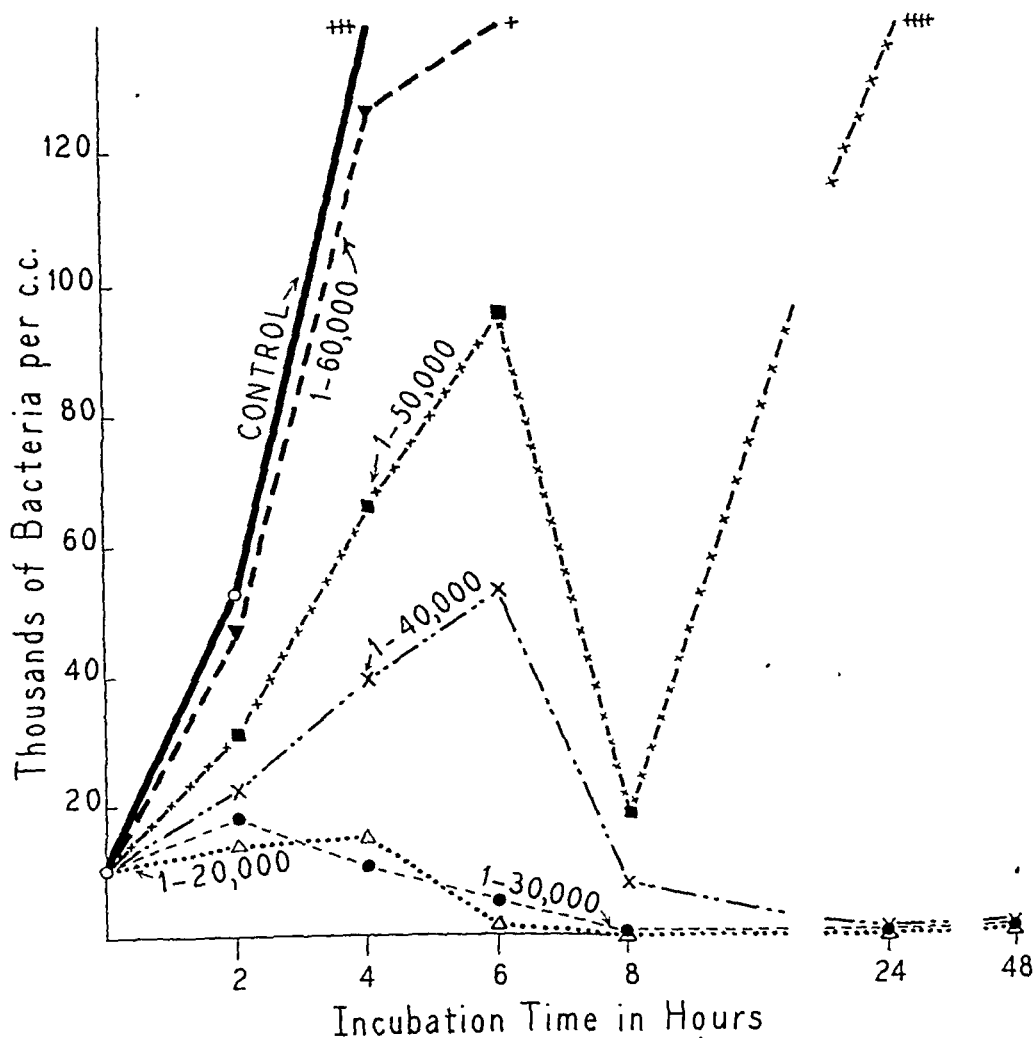


Fig. 6.—Antibacterial action of protoanemonin.

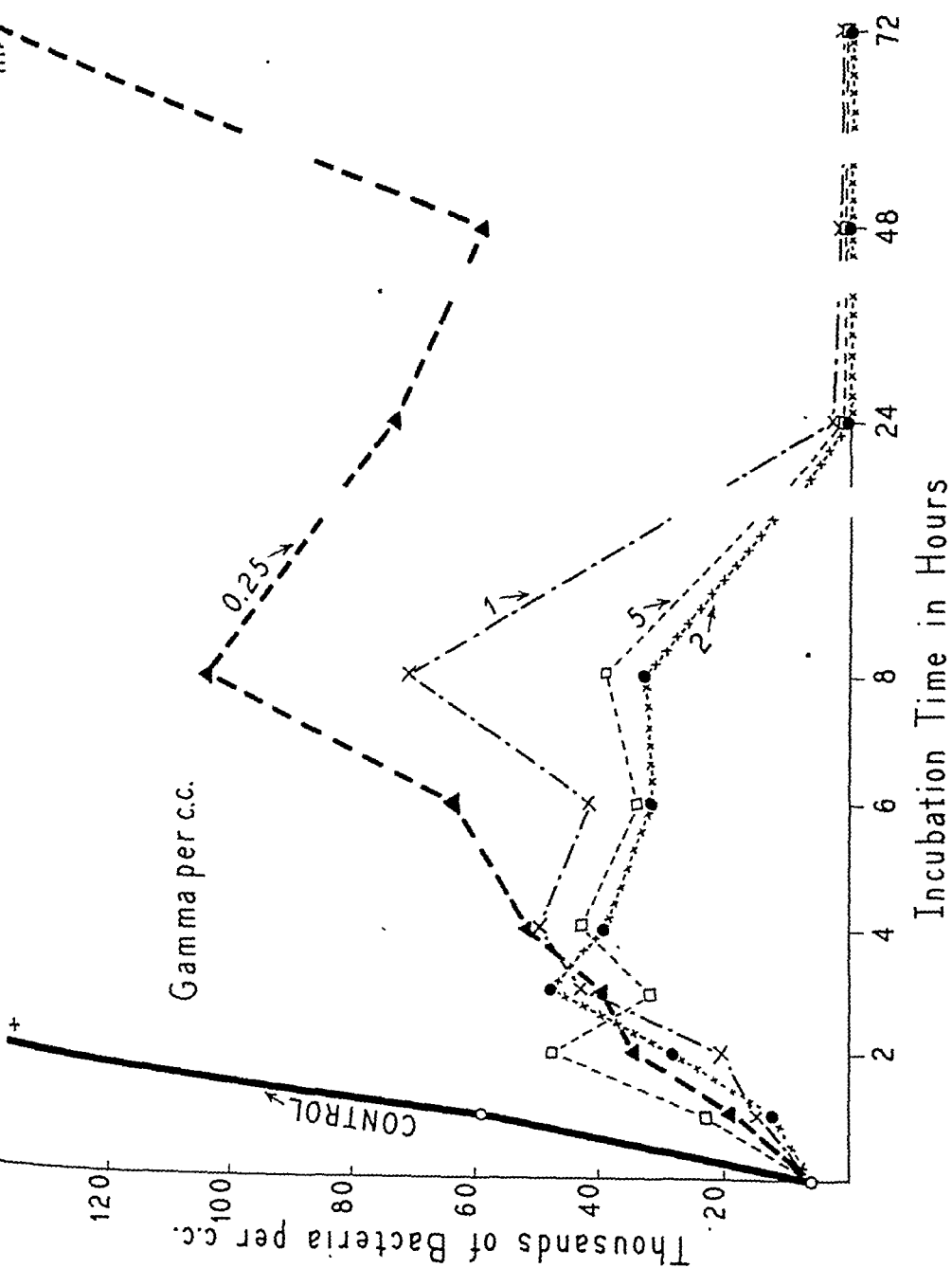


Fig. 7.—Antibacterial action of Mn. protoporphyrin for *Staph. aureus* (gamma per cubic centimeter).

irreversible, nor was it proved that subculture of a larger sample might not have shown a few surviving cells.

It was evident, however, that the effect of phenol was purely bactericidal in the usually accepted sense. High concentrations of streptothricin, streptomycin,\* and penicillin also appeared bactericidal within two hours. A slower

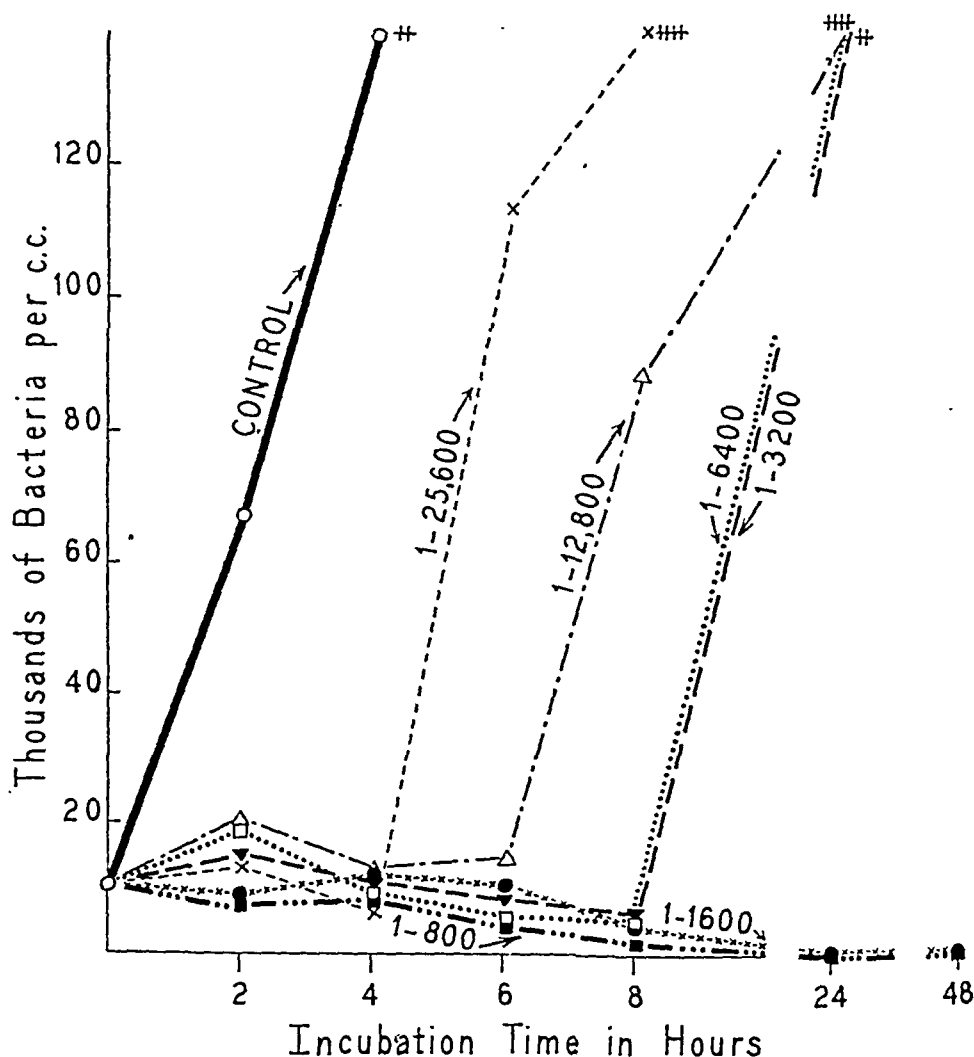


Fig. 8.—Antibacterial action of alcohol solution of sulfur (alcohol-sulfur) for *Staph. aureus*.

bactericidal effect is shown by clavacin and protoanemonin in six and eight hours, respectively. The other three antibacterial agents (Mn. protoporphyrin, alcohol-sulfur, and carbowax-sulfur), even in the highest concentrations used, showed no bactericidal effect during the first eight-hour period of growth.

\*The 0.2 unit curve which gave negative cultures at one, two, four, six, eight, twenty-four, and forty-eight hours is omitted from Fig. 3.

# STREAK PLATE METHOD FOR GROWTH CURVES

Sulfanilamide also showed no bactericidal effect, but this may have been due to its insolubility in sufficiently high concentration. Adequate test of pentathionic acid could not be made because high concentrations were too acid to permit growth of the staphylococcus.

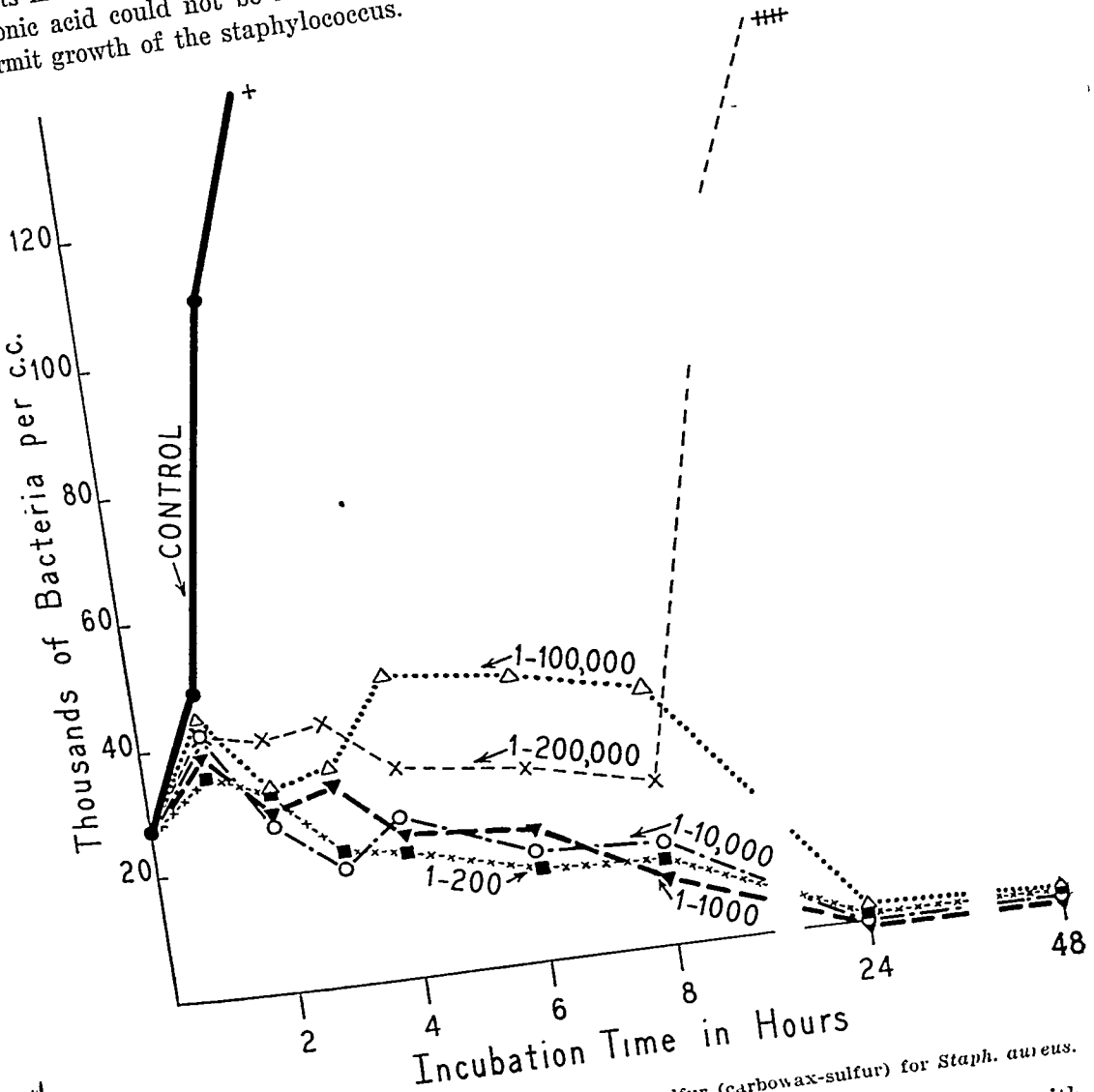


Fig. 3.—Antibacterial action of carbowax solution of sulfur (carbowax-sulfur) for *Staph. aureus*.

2. *The Phase of Early Growth Followed by Inhibition.*—This occurs with intermediate concentrations of several of the antibacterial agents tested. It is seen at approximately two hours with streptothricin. A more prolonged growth phase with a peak of growth at six hours is shown by protoanemonin and sulfanilamide. Mn. protoporphyrin differs from streptothricin and protoanemonin in that it permitted rapid early growth with peaks at four to eight hours. The other antibacterial agents tested show no definite early phase of growth.

3. *Bacteriostatic Effect*.—All agents tested showed a zone in which the cocci were inhibited for a period but began active growth within eight hours. With phenol the period lasted only two hours in a narrow zone of concentration. With protoanemonin, clavaein, and penicillin the range of concentration showing only inhibition was also narrow. It was somewhat wider in streptothricin, streptomycin, and Mn. protoporphyrin. The curves for sulfanilamide also

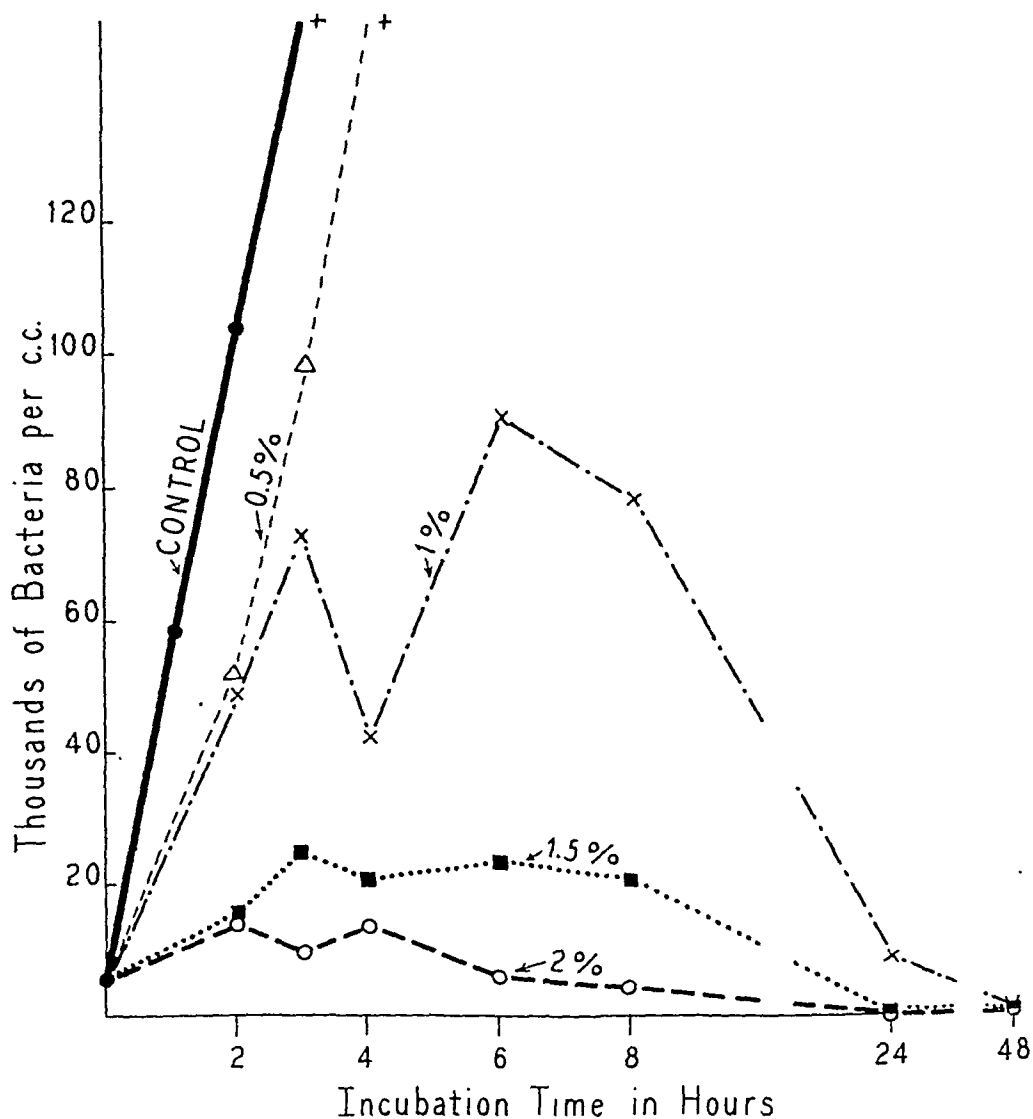


Fig. 10.—Antibacterial action of sulfanilamide for *Staph. aureus*.

showed inhibition only in a narrow zone, but this may have been due to the presence of sulfonamide antagonists in the medium which counteracted its effect except in high concentration. The low pH of strong solutions of pentathionic acid also prevented determination of its range of activity.

With the sulfur suspensions, inhibition seemed of a different order. In these there was an enormous range of concentrations in which the organisms were held at about the inoculation level for from four to eight hours, only to grow out freely at a later period. Tubes with higher concentrations in which the organisms were inhibited for twenty-four hours or longer appeared sterile on transfer. It seemed more probable that this was due to senescence of the inhibited organisms rather than to direct bactericidal effect.<sup>8</sup>

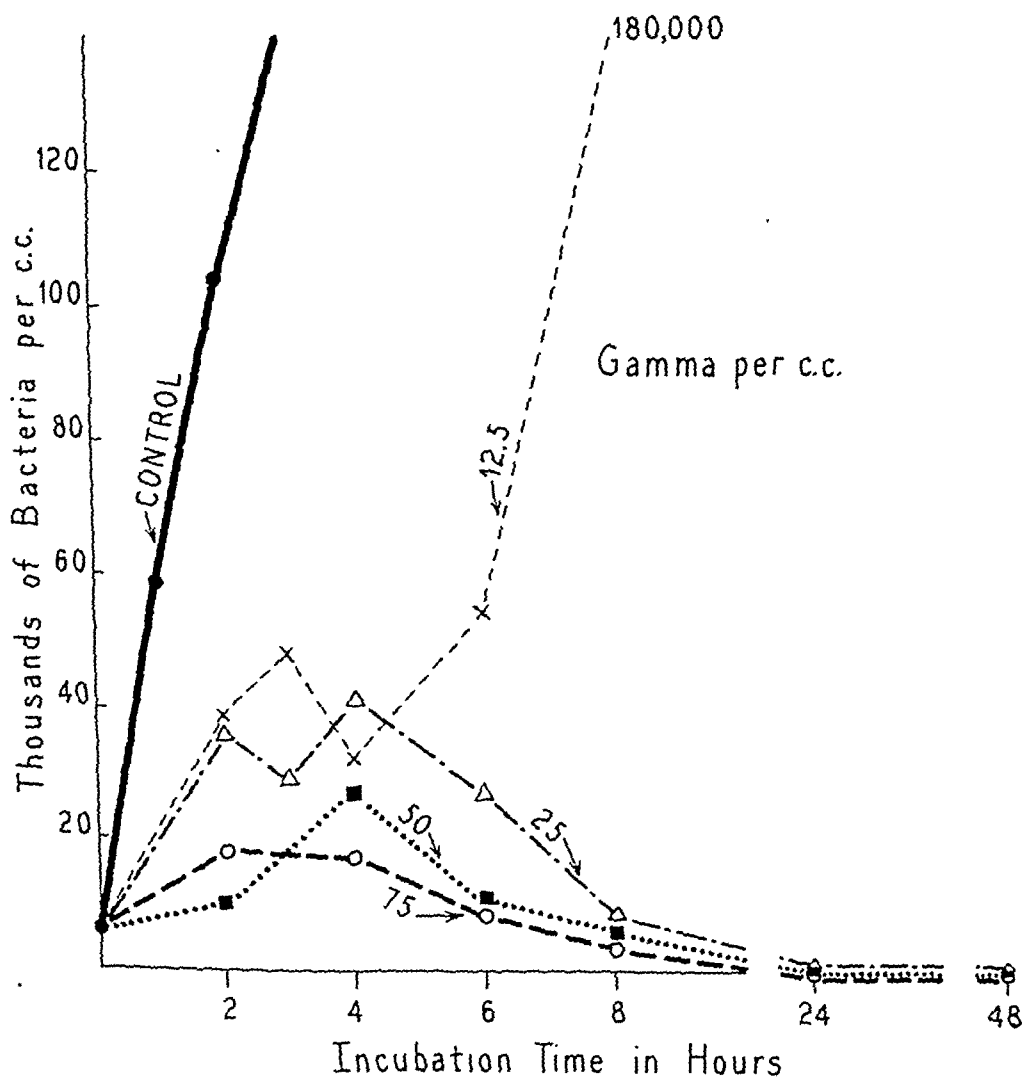


Fig. 11.—Antibacterial action of pentathionic acid for *Staph. aureus* (gamma per cubic centimeter).

4. *The Renewal of Growth Phenomenon.*—This was seen after eight to twenty-four hours' growth, with intermediate concentrations of several antibacterial substances after the organisms had practically disappeared from the cultures and appeared to be dying out. This was probably due to the presence

of a small number of resistant organisms in the test culture.<sup>9-11</sup> This phenomenon is most apparent with streptothricin and streptomycin and, to a less extent, with clavacin and possibly with Mn. protoporphyrin. It was not seen in the sulfur preparations or with sulfanilamide. In concentrations of these latter agents, which inhibited the staphylococci for eight hours, the organisms failed to grow out after longer exposure.

Table I demonstrates that the substances tested may be separated roughly into two groups:

1. Antibacterial agents which are bactericidal in eight hours or less. In this group the minimum bactericidal dose is only 40 or less times the maximum noninhibiting dose. The range of dilutions which gives inhibition without bactericidal effect is small.

2. Antibacterial agents which are merely bacteriostatic in eight hours. This group gives only bacteriostasis in doses as high as 80 to 8,000 times the maximum noninhibiting dose. The range of dilutions which gives inhibition is large.

#### SUMMARY

A simple streak plate method for demonstrating growth curves of *Staph. aureus* under the influence of antibacterial agents is described.

Graphs are presented showing the effect of the following antibacterial agents:

*Phenol*: A rapidly bactericidal substance which kills the organisms in a concentration only slightly higher than one which has even no inhibiting effect.

*Streptothricin, Streptomycin, Clavacin, Penicillin and Protoanemonin*: Antibacterial agents which kill the organisms in eight hours or less in concentrations not more than 40 times the noninhibiting doses.

*Mn. protoporphyrin and Suspensions of Sulfur in Alcohol (Alcohol-Sulfur) and in Carbowax (Carbowax-Sulfur)*: Purely bacteriostatic preparations which fail to kill the organisms in eight hours in the highest concentrations tested but inhibit growth completely in minute fractions of such amounts.

#### REFERENCES

1. Weld, Julia T., and Gunther, Anne: The Antibacterial Properties of Sulfur, *J. Exper. Med.* 85: 531, 1947.
2. Calkins, F. C.: Simple Devices for Measuring Out Small Quantities of Liquid, *J. Chem. Education* 23: 604, 1946.
3. McClelland, C. P., and Bateman, R. L.: Technology of the Polyethylene Glycols and Carbowax-Compounds, *Chem. and Eng. News* 23: 247, 1945.
4. Seegal, Beatrice C., and Holden, Margaret: The Antibiotic Activity of Extracts of *Ranunculaceae*, *Science* 101: 413, 1945.
5. Baer, Harold, Holden, Margaret, and Seegal, Beatrice: The Nature of the Antibacterial Agent From *Anemone Pulsatilla*, *J. Biol. Chem.* 162: 65, 1946.
6. Granick, S., and Gilder, H.: Personal communication.
7. Liming, O. N.: The Preparation and Properties of Pentathionic Acid and Its Salts; Its Toxicity to Fungi, Bacteria, and Insects, *Phytopathology* 23: 155-170, 1933.
8. Topley, W. W. C., and Wilson, G. S.: Principles of Bacteriology and Immunity, ed. 3, Baltimore, 1946, vol. 8, Williams & Wilkins Company.
9. Demerick, M.: Production of *Staphylococcus* Strains Resistant to Various Concentrations of Penicillin, *Proc. Nat. Acad. Sc.* 31: 16, 1945.
10. DuBois, A. S., and Dibblee, Diana: Death-rate Study on the High Molecular Quaternary Ammonium Compound With *Bacillus Metiens*, *Science* 103: 734, 1946.
11. Alexander, H. H., and Leidy, G.: Mode of Action of Streptomycin on Type b H. Influenzae, *J. Exper. Med.* 85: 329, 1947.



# FLUID THIOLYCOLLATE MEDIUM

## ITS USE FOR TESTING THE STERILITY OF SURGICAL CATGUT SUTURES

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BREWER,<sup>1</sup> in 1940, announced his fluid thioglycollate medium for the cultivation of anaerobes and stated that it also would support growth of aerobes. In his table, he showed that *Clostridium novyi* would grow satisfactorily up to a month after sterilization of the culture medium, but he did not mention the size of the inoculum used. He stated in his paper: "The sodium thioglycollate combines with and inactivates most of the mercurials used as preservatives, and one is much more likely to obtain growth from contaminated biologicals which are preserved with one of these highly bacteriostatic compounds."

McClung<sup>4</sup> reported that Brewer's thioglycollate medium compares favorably with complex meat infusions in its ability to initiate growth from small numbers of cells. For his tests, eleven different species of anaerobes including *Cl. novyi* were used.

Nungester, Hood, and Warren<sup>6</sup> conducted experiments to determine whether the addition of sodium thioglycollate to culture medium used in testing disinfectants by an in vitro method would give results similar to those obtained by the "infection-prevention" method. They experimented with six mercurial compounds and found the percentage of positive growths was much greater when a culture medium containing thioglycollate was used. They concluded: "Sodium thioglycollate added to media for testing disinfectants *in vitro* apparently neutralized the bacteriostatic effect of the mercurial compounds tested and thus indicated the inadequacy of these substances as germicides."

As a result of Brewer's findings, that the addition of sodium thioglycollate to culture medium inhibits the bacteriostatic action of mercurials, the National Institute of Health<sup>5</sup> specified a formula for fluid thioglycollate medium which it recommends for use in testing the sterility of biologic products. This culture medium<sup>8</sup> is a modification of the original Brewer formula.

It has been suggested that fluid thioglycollate medium might be used effectively to test the sterility of all products that are preserved or impregnated with mercurial compounds. Such a procedure would utilize the fluid thioglycollate medium for testing the sterility of surgical catgut sutures instead of the culture media officially prescribed by the *United States Pharmacopoeia XII*.

### PURPOSE OF INVESTIGATION

The purpose of this investigation was twofold: (1) to determine the comparative growth-promoting properties of the National Institute of Health formula for fluid thioglycollate medium with those of the Novy culture medium which is specified in the U. S. P. XII as the official anaerobic medium, and (2)

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\*Prepared by the Difco Laboratories, Incorporated, Detroit, Mich.

to determine the efficiency of the fluid thioglycollate medium for testing the sterility of surgical catgut sutures *without* employing the inactivating fluids which constitute part of the U. S. P. XII Sterility Tests for Solids.

#### MATERIALS AND METHODS

The culture media comprised (1) bacto-fluid thioglycollate medium and (2) the U. S. P. XII medium prepared for aerobiosis and that prepared for anaerobiosis. The fluid thioglycollate medium was incubated without seal, while tubes of the U. S. P. XII anaerobic medium were sealed with a mixture of equal parts of petroleum jelly and paraffin. The final pH of the fluid thioglycollate medium was adjusted to 7.1 as recommended by Pittman,<sup>7</sup> while that of the U. S. P. XII media was adjusted to 7.4 as specified in the U. S. P. XII.

A series of experiments was conducted to determine the growth-promoting properties of the fluid thioglycollate medium and of the U. S. P. XII anaerobic medium, using *Cl. novyi* as the test organism in decimal dilutions. In the official Sterility Tests for Solids, the U. S. P. XII specifies<sup>8</sup> *Cl. novyi* for the control organism to determine the growth-promoting properties of the culture medium.

Another series of experiments involved the testing for sterility of catgut sutures artificially infected with a sporulating culture of *Clostridium sporogenes* and *Bacillus subtilis*, and impregnated with mercuric iodide to the amount of 1 per cent in some of the sutures and 3.5 per cent in others.

#### EXPERIMENTS TO DETERMINE GROWTH-PROMOTING PROPERTIES

Several tubes of the fluid thioglycollate medium and of the U. S. P. XII anaerobic medium were inoculated with 1 c.c. of decimal dilutions (from  $10^{-1}$  to  $10^{-10}$ ) of a forty-eight hour broth culture of *Cl. novyi*. Typical protocols are shown in Table I. This experiment was repeated several times and the results were similar to those shown in the table.

#### Discussion of Results.—

1. *Fluid Thioglycollate Medium*: After twenty-four hours gas formed in the first dilution, and a very light bacterial growth occurred from an inoculum of 0.000001 c.c. ( $10^{-6}$ ).

After forty-eight hours gas formed in the first five tubes.

After five days maximum degree of growth (4 plus) occurred from an inoculum of 0.00001 c.c. ( $10^{-5}$ ), gas formed from an inoculum of 0.000001 c.c. ( $10^{-6}$ ), and a very light bacterial growth was present in the tube containing an inoculum of 0.00000001 c.c. ( $10^{-8}$ ).

2. *U. S. P. XII Anaerobic Medium*: After twenty-four hours, a very light bacterial growth was present in the tube containing an inoculum of 0.00001 c.c. ( $10^{-5}$ ), but gas did not form in any of the tubes.

After forty-eight hours gas formed in the first four tubes.

After five days maximum degree of growth (4 plus) occurred from an inoculum of 0.001 c.c. ( $10^{-3}$ ), gas formed from an inoculum of 0.00001 c.c. ( $10^{-5}$ ), and a light bacterial growth occurred from an inoculum of 0.000001 c.c. ( $10^{-6}$ ).

TABLE I

MEDIUM	INCUBATION PERIOD	DECIMAL DILUTIONS									
		10-1	10-2	10-3	10-4	10-5	10-6	10-7	10-8	10-9	10-10
Bacto-fluid thioglycollate medium	24 hr.	4+ gas	3+	3+	2+	2+	+	0	0	0	0
	48 hr.	4+ gas	4+ gas	4+ gas	3+ gas	3+ gas	2+	+	0	0	0
	5 days	4+ gas	4+ gas	4+ gas	4+ gas	4+ gas	3+	2+	+	0	0
		4+ gas	3+	2+	+	+	0	0	0	0	0
U. S. P. medium for anaerobiosis	24 hr.	4+	3+	2+	+	+	0	0	0	0	0
	48 hr.	4+ gas	4+ gas	3+ gas	3+ gas	2+ gas	+	0	0	0	0
	5 days	4+ gas	4+ gas	4+ gas	3+ gas	3+ gas	2+	0	0	0	0
		4+ gas	4+ gas	4+ gas	4+ gas	4+ gas	4+ gas	4+ gas	4+ gas	4+ gas	4+ gas

The degree of bacterial growth is indicated by plus signs: very light cloud, 1 plus; light cloud, 2 plus; heavy cloud, 3 plus; and very heavy cloud, 4 plus. Absence of growth is indicated by 0.

Decimal dilutions of a forty-eighth and the U. S. P. anaerobic medium. In the tubes of the U. S. P. anaerobic medium, bacterial growth was present on the fifth day in the tubes of bacto-fluid thioglycollate medium as far out as the eighth dilution in contrast to the sixth dilution in the tubes of the U. S. P. anaerobic medium.

These results indicate that the fluid thioglycollate medium is superior to the U. S. P. XII anaerobic medium for supporting the growth of anaerobic bacteria, such as *Cl. novyi*. This superiority is shown (1) after twenty-four hours' incubation, by gas formation in the first dilution and by bacterial growth out as far as the sixth dilution; (2) after forty-eight hours' incubation, by the maximum degree of growth occurring in one higher dilution and by bacterial growth being present in one higher dilution; and (3) after five days' incubation, by gas formation in one higher dilution, by the maximum degree of bacterial growth occurring in two dilutions higher, and by bacterial growth being present out as far as the eighth dilution in contrast to the sixth dilution.

#### EXPERIMENTS ON INACTIVATION OF MERCURIALS

To determine the extent of the combining and inactivating properties of thioglycollic acid (contained in the fluid thioglycollate medium) for mercury, a series of experiments was carried out in which artificially infected catgut sutures containing 1 per cent mercuric iodide (Lot 1754) and some containing 3.5 per cent mercuric iodide (Lot 1755) were tested for sterility. The sutures were infected with a sporulating culture of *Clostridium sporogenes* and *Bacillus subtilis*. For controls, sutures artificially infected with the sporulating culture but not impregnated with any chemical compound (Lot 1753) were used.

Comparative tests were made by placing some of each of the three different lots of sutures directly into tubes containing 40 c.c. fluid thioglycollate medium and incubating at 37° C.; other sutures of each of the three lots were tested in accordance with the sterility technique prescribed by the U. S. P. XII. This latter technique consisted of incubating the sutures first in tubes of 40 c.c. distilled water, then in tubes containing the inactivating fluid of 1 per cent

sodium thiosulfate and 1 per cent sodium carbonate; on the third day the sutures were transferred to tubes of distilled water and incubated twenty-four hours; and on the fourth day the sutures were transferred to tubes of beef broth prepared for aerobiosis and incubated, or transferred to tubes of gelatin beef broth prepared for anaerobiosis and sealed with a mixture of petroleum jelly and paraffin and then incubated. Results of these comparative tests are shown in Tables II and III.

TABLE II

LOT	BACTO-FLUID THIOLYCOLLATE MEDIUM	
	AEROBIC RESULTS	ANAEROBIC RESULTS
1753 (Control)	24-hour growth, gram-positive rods	24-hour growth; gram positive rods with subterminal spores; gas formation
1754 (Containing 1% mercury)	24-hour growth, gram-positive rods	24 hour growth: gram positive rods with subterminal spores; gas formation
1755 (Containing 3.5% mercury)	24 hour growth, gram-positive rods	24 hour growth: gram-positive rods with subterminal spores; gas formation

The inactivating properties of bacto-fluid thioglycollate medium for mercury were determined by sterility tests of eight sutures artificially infected with a sporulating culture. Some sutures contained 1 per cent mercury, while others with 1.5 per cent mercury. For controls, sutures artificially infected but not with mercury were used. These tests show that the thioglycollate acid in the bacto-fluid thioglycollate medium combines with and inactivates 1 per cent mercury and 3.5 per cent mercury, so that twenty-four hour growths occurred in all tubes and corresponded to the growths in the control tubes.

The results given in Table II indicate that the thioglycollic acid in the fluid thioglycollate medium combines with and inactivates the 1 per cent of mercury and the 3.5 per cent of mercury, as shown by the aerobic and anaerobic bacterial growths which occurred in Lots 1754 and 1755 and which checked with the control Lot 1753.

Table III shows the results of using the U. S. P. XII technique. It will be noted that the mercurial salts present in the sutures to the amount of 1 per cent combine with the sodium thiosulfate of the inactivating fluid to form a double salt which is soluble in water, as shown by the aerobic and anaerobic bacterial growths in Lot 1754. However, the amount of mercury in sutures of Lot 1755 is so great (3.5 per cent) that all of it will not combine with the sodium thiosulfate of the inactivating fluid, and enough mercury is still present in the sutures to exert a bacteriostatic effect and prevent bacterial growth.

TABLE III

LOT	U.S.P. AEROBIC MEDIUM	U.S.P. ANAEROBIC MEDIUM
	AEROBIC RESULTS	ANAEROBIC RESULTS
1753 (Control)	24 hour growth, gram positive rods	24 hour growth: gram positive rods with subterminal spores; gas formation
1754 (Containing 1% mercury)	24 hour growth, gram-positive rods	24 hour growth: gram-positive rods with subterminal spores; gas formation
1755 (Containing 3.5% mercury)	No growth after 15 days' incubation	No growth after 15 days' incubation

Experiments were made to determine the inactivating properties for mercury of the standard inactivating fluid of 1 per cent sodium thiosulfate and 1 per cent sodium carbonate prescribed by the U. S. P. XII. Catgut sutures artificially infected and containing 1 per cent mercury and some containing 3.5 per cent mercury were tested for sterility by the U. S. P. XII technique. For controls, sutures artificially infected but not impregnated with mercury were used. These tests show that the standard inactivating fluid combines with and inactivates 1 per cent mercury (as shown by twenty-four hour growths in Lot 1754), but that it is not capable of combining with and inactivating 3.5 per cent mercury (as shown by the absence of growth in Lot 1755).

In my bacteriologic investigations of chemical sterilization of surgical catgut sutures, I devised<sup>2</sup> an inactivating fluid of 10 per cent sodium thiosulfate for effectively combining with and inactivating mercurial compounds if present in amounts greater than 2 per cent. I recommended<sup>3</sup> the use of this 10 per cent sodium thiosulfate solution as an inactivating fluid whenever catgut sutures are found upon chemical analyses to contain more than 2 per cent of mercury. The U. S. P. XII adopted<sup>8</sup> this procedure as part of the official Sterility Tests for Solids.

Accordingly, additional sutures from Lot 1755 were tested by the U. S. P. XII technique in which the 10 per cent sodium thiosulfate inactivating fluid was used, with results shown in Table IV.

TABLE IV

LOT	U.S.P. AEROBIC MEDIUM	U.S.P. ANAEROBIC MEDIUM
	AEROBIC RESULTS	ANAEROBIC RESULTS
1753 (Control)	Growth in 24 hours, gram-positive rods	Growth in 24 hours: gram-positive rods with subterminal spores; gas formation
1755 (Containing 3.5% mercury)	Growth in 24 hours, gram-positive rods	Growth in 24 hours: gram-positive rods with subterminal spores; gas formation

Experiments were made to determine the inactivating properties of the special inactivating fluid of 10 per cent sodium thiosulfate prescribed in the U. S. P. XII sterility technique when large amounts of mercury are present in sutures. Catgut sutures artificially infected with a sporulating culture and containing 3.5 per cent mercury were tested for sterility. Sutures artificially infected but not impregnated with mercury were used for controls. These tests show that the special inactivating fluid of 10 per cent sodium thiosulfate combines with and inactivates 3.5 per cent mercury, so that in all tubes of Lot 1755 growths occurred in twenty-four hours, which corresponded to growths in the controls.

These results demonstrate that the special inactivating fluid of 10 per cent sodium thiosulfate is required in connection with the U. S. P. XII technique in order to detect the presence of living bacteria in catgut sutures which are heavily impregnated with mercurial salts. This special inactivating fluid eliminates all bacteriostatic action of the mercury, thereby allowing the bacteria in the catgut to germinate in the culture medium.

*Discussion of Results.*—This series of experiments to determine the inactivation of mercurials demonstrates in a convincing manner that the fluid thioglycollate medium is superior to the U. S. P. XII culture media for testing the sterility of surgical catgut sutures. It simplifies the technique because it may be used *without* the inactivating fluids specified in the U. S. P. XII for the inactivation of mercurial salts. The thioglycollic acid contained in the fluid thioglycollate medium combines with the mercury and thereby eliminates all bacteriostatic action of mercurial salts when present in catgut sutures in amounts as great as 3.5 per cent.

#### CONCLUSIONS

The experiments herein described demonstrate that bacto-fluid thioglycollate medium is superior, in the following respects, to the U. S. P. XII culture media for testing the sterility of surgical catgut sutures:

1. A smaller inoculum supports the growth of anaerobic bacteria, such as *C. novyi*.
2. Gas forms earlier, and maximum bacterial growth occurs from a much smaller inoculum.

3. No seal is required for anaerobiosis, since the sodium salt of thioglycollic acid lowers the oxidation reduction potential which is maintained for a considerable period of time by the agar in the medium.

4. Preliminary incubation in distilled water and inactivating fluids, for the purpose of removing bacteriostatic mercurial salts with which the sutures may be impregnated, is not required.

5. The sodium salt of thioglycollic acid in the culture medium effectively neutralizes the bacteriostatic action of mercurial compounds when present in catgut sutures in amounts up to 3.5 per cent.

Since these experiments were completed, the Committee of Revision of the United States Pharmacopoeia adopted the National Institute of Health formula for fluid thioglycollate medium for use in Sterility Tests for Liquids and Solids, and it will become the official sterility test medium in the U. S. P. XIII.

#### REFERENCES

1. Brewer, J. H.: Clear Liquid Mediums for the Aerobic Cultivation of Anaerobes, *J. A. M. A.* 115: 598, 1940.
2. Clock, R. O.: The Fallacy of Chemical Sterilization of Surgical Catgut Sutures, *Surg., Gynec. & Obst.* 56: 149, 1933.
3. Clock, R. O.: A Reliable Method for Testing the Sterility of Surgical Catgut Sutures, *Surg., Gynec. & Obst.* 61: 789, 1935.
4. McClung, L. S.: The Use of Dehydrated Thioglycollate Medium in the Enrichment of Spore-Forming Anaerobic Bacteria, *J. Bact.* 40: 645, 1940.
5. Culture Media for the Sterility Test, National Institute Health Circular, Second Revision. Feb. 5, 1946.
6. Nungester, W. J., Hood, M. N., and Warren, M. K.: Use of Thioglycollate Media for Testing Disinfectants, *Proc. Soc. Exper. Biol. & Med.* 52: 287, 1943.
7. Pittman, M.: A Study of Fluid Thioglycollate Medium for the Sterility Test, *J. Bact.* 51: 19, 1946.
8. United States Pharmacopoeia XII, 1942, p. 610; Sterility Tests for Solids.

# DETERMINATION OF INULIN IN PLASMA AND URINE

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THE use of inulin in measuring glomerular filtration has stimulated the development of a great number of methods for the estimation of this substance in plasma and urine. Among the reagents used, diphenylamine appears to yield the greatest color density per milligram of inulin present. However, even employing this reagent in Harrison's\* modification of the method of Alving, Rubin, and Miller, we found that the color produced by low concentrations of inulin (4 to 20 mg. per cent) proved to be too feeble to permit exact readings in the Pulfrich photometer. Since it is known that the color intensity obtained with diphenylamine is greatly reduced in the presence of water, we attempted to find a method for precipitating inulin from the plasma filtrates and for subsequently carrying out the estimation with the dried precipitate. Such a method would have an additional advantage in that larger aliquots could be analyzed than have been possible with current methods.

In preliminary experiments it was found that inulin can be quantitatively precipitated from solution by treatment with calcium hydroxide. No sugar can be detected in the supernatant fluid, nor is any sugar eluted by subsequent washing of the precipitate with alkaline water or with alcohol. On addition of the strongly acid diphenylamine reagent, calcium hydroxide is easily dissolved, setting free the precipitated inulin for hydrolysis and color production.

The following method was evolved for the determination of inulin in blood and urine as part of the measurement of inulin clearance in man.

## METHOD

### *Solutions.*—

Zinc sulfate solution 10 per cent.

Sodium hydroxide solution N/2.

Calcium chloride solution 2N.

Sodium hydroxide solution 2N.

Saturated solution of calcium hydroxide in water.

Saturated solution of calcium hydroxide in alcohol.

Diphenylamine reagent. 3 Gm. diphenylamine dissolved in 100 ml. glacial acetic acid. Add 60 ml. concentrated hydrochloric acid. Store in amber bottle in a cold place.

Inulin stock solution. 100 mg. inulin in 100 ml. water containing 1 drop of N/10 sodium hydroxide. This solution keeps for two weeks in a cold place.

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\*Harrison, H. E.: A Modification of the Diphenylamine Method for Determination of Inulin, *Proc. Soc. Exper. Biol. & Med.* 49: 111, 1942.

Working standard. Freshly prepared from the stock solution by diluting 2 to 100 ml. with water.

*Procedure.*—Measure 0.2 to 0.5 ml. of plasma or serum containing 10 to 100  $\mu$ g of inulin into a centrifuge tube. Make up to 9 ml. with water, add 0.5 ml. zinc sulfate solution, and mix. Add 0.5 ml. of N/2 sodium hydroxide solution, mix, centrifuge, and filter. Measure 5 ml. of the clear filtrate into a centrifuge tube and add 0.5 ml. calcium chloride solution and 0.5 ml. 2N sodium hydroxide solution. Heat in a boiling water bath for five minutes. Centrifuge sharply for five minutes. Decant the supernatant fluid. Wash the precipitate with 5 ml. of the aqueous solution of calcium hydroxide, using a small glass rod for each tube in order to break up the larger clumps. Put the rods aside for further use with their respective tubes. Centrifuge the suspension sharply, decant, and wash with alcohol-calcium hydroxide as described. After decantation drive off the remaining alcohol by immersing the tubes in a boiling water bath for five minutes. Return the rods to their tubes. Add 5 ml. of diphenylamine reagent, stir well, and heat in a boiling water bath for exactly thirty minutes. Cool in cold water. Read in a photometer with a filter transmitting at 610 millimicrons.

Each run includes one plasma blank from the patient's blood before the injection, and two standards, prepared by adding 0.5 ml. of the blank plasma to 2 ml. and 4 ml. of the standard inulin solution, making up to 9 ml., and proceeding as described.

Urine is diluted 500- to 1,000-fold. To 5 ml. of the diluted urine add 0.5 ml. of calcium chloride solution, and proceed as with the blood filtrates. A blank value is determined from the patient's urine before the test.

*Calculation.*—The extinction values obtained with the two standards included in the series give the factors needed for calculation of both plasma concentration and urinary excretion.

#### DISCUSSION OF METHOD

*Color Intensity.*—With this method, 10  $\mu$ g of inulin in the final solution gave an extinction of 0.2 with a 10 mm. cell. This value was practically constant. We do not, however, propose to dispense with the inclusion of standards in each run, since changes might occur in heating conditions or in various reagent batches which would otherwise remain undetected.

Our method achieves color intensities five times as strong as those obtained with Harrison's method (Table I). This is accounted for by our use of 5 ml. of

TABLE I. COMPARISON OF METHODS

INULIN IN PLASMA (MG. %)	EXTINCTION IN 1 CM. CELL MINUS BLANK	
	ACCORDING TO HARRISON'S METHOD	ACCORDING TO OUR METHOD
1	0.04	0.20
8	0.08	0.40
20	0.18	1.04



TABLE II. RECOVERY OF INULIN ADDED TO PLASMA

PLASMA (ML.)	INULIN ADDED (MG. %)	INULIN RECOVERED (MG. %)
0.5	4.0	4.4
-	8.0	8.2
-	16.0	16.0
-	4.0	4.0
-	8.0	7.6
-	16.0	16.6
-	5.0	5.2
-	10.0	10.4
-	20.0	19.4
0.2	4.0	3.5
-	8.0	7.8
-	16.0	16.5
-	4.0	4.0
-	8.0	8.0
-	16.0	16.0
-	25.0	24.0

blood filtrate instead of 2 ml. and by the exclusion of water, giving an additional reduplication of color.

*Precipitation of Inulin.*—The amount of precipitant used was found to allow for quantities of inulin in plasma filtrates up to 100 micrograms. Attempts to dispense with the boiling for precipitation have yielded erratic results. Heat seems to be necessary to insure a stable relation between inulin and calcium hydroxide which will withstand subsequent washing.

*Blanks.*—The diphenylamine reagent develops a color with ageing and should be frequently prepared in small quantities. We found the urine blank values to be no higher than those of the reagent blank with water. A water blank may, therefore, be substituted for the urine blank proposed if no sugar or other abnormal constituents are present which produce color with the reagent. Serum blank values were equivalent to about 4 to 5 mg. per cent inulin. The same extinction was obtained by treating a solution of 100 mg. per cent glucose in water as described for plasma. It is thus mainly glucose which is responsible for the serum blank value. The proportion of 1:25 in color produced by glucose and inulin, respectively, would require a fluctuation in blood sugar of 25 mg. per cent in order to change the apparent inulin content by 1 mg. per cent. We consider ourselves, therefore, justified in dispensing with yeast treatment, except under very special experimental conditions.

*Recovery Experiments.*—Recovery experiments are presented in Table II.

*Application of the Method for Inulin Clearance Tests.*—With the described method for inulin determination, we found it entirely sufficient to inject only 1.5 or 2 Gm. intravenously instead of the usual 10 grams. This dose gives an initial serum level of 12 to 15 mg. per cent with sufficient time for several clearance periods before the concentration has fallen too low for determination. This should make inulin clearance studies more attractive to clinicians, who have been reluctant to undertake them because of the danger of pyrexial reactions. The higher color production makes clearance studies feasible even in small laboratory animals by reducing the quantity of plasma required for analysis.

## SUMMARY

A method has been developed for the estimation of inulin in blood plasma and in urine. Inulin is precipitated with calcium hydroxide and the color elicited with the dry precipitate with diphenylamine. This increases greatly the color intensity obtained.

The method permits the reduction of the inulin dose injected for clearance tests in human beings and makes clearance tests feasible in small laboratory animals.

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# CHROMATOGRAPHIC SEPARATION OF CHOLESTEROL AND CHOLESTEROL ESTERS IN BLOOD

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THE methods commonly used for the determination of free cholesterol in blood depend upon its precipitation with digitonin followed by the development of color directly upon the digitonide, usually with the Liebermann-Burchard procedure.<sup>1-3</sup> The estimation of cholesterol esters, which are not precipitated by digitonin, should be done after saponification because, as has been repeatedly shown, the esters give more color in the Liebermann-Burchard method than does free cholesterol.<sup>4-8</sup> In the estimation of both free and ester cholesterol these two methods are combined.<sup>2, 3</sup> For complete precipitation of the cholesterol digitonide twenty-four hours are required, although Schoenheimer and Sperry<sup>3</sup> state that satisfactory results can be obtained with only one-hour standing. A method which would eliminate the costly and time-consuming digitonin precipitation is to be desired.

Trappe<sup>9</sup> used aluminum oxide to adsorb various lipids from petroleum ether solution and separated cholesterol stearate from free cholesterol by first eluting with trichlorethane to remove the ester and then with absolute ethyl alcohol to remove the cholesterol. He used large amounts of material, 71.2 mg. of cholesterol stearate and 72.2 mg. of cholesterol, and recovered 99.3 per cent of the stearate despite the fact that there was some saponification of the ester. A satisfactory method for blood analysis would require the separation and determination of much smaller quantities of cholesterol ester and free cholesterol. Subsequently Trappe<sup>10, 11</sup> described a method for the determination of both free and ester cholesterol in whole blood based, in part, upon these experiments. His procedure involved the use of both silica gel and aluminum oxide; however, no values for either plasma or serum were given.

Chromatographic procedures have been used in our laboratory for the separation of estrone from blood<sup>12</sup> and it seemed possible to devise a similar method for the determination of both free and ester cholesterol. This paper describes such a method based upon adsorption on aluminum oxide and upon selective elution followed by estimation by the Liebermann-Burchard method.

## METHOD

*Principle of the Method.*—Either plasma or serum is treated with a 1:1 acetone-ethyl alcohol solution and filtered. An aliquot of the filtrate is evaporated to dryness dissolved in petroleum ether, and placed upon an aluminum oxide column. The ester fraction is eluted with a 10 per cent solution of ethyl ether in petroleum ether, and the free cholesterol is then eluted with a 10 per cent solution of ethyl alcohol in petroleum ether. The cholesterol esters are then saponified and the free cholesterol in both fractions is determined colorimetrically, using cholesterol as the standard.

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*Procedure.*—To approximately 20 ml. of a 1:1 acetone-ethyl alcohol solution in a 25 ml. volumetric flask add 1.0 ml. of plasma or serum. The suspension is brought to boiling on the water bath, cooled, and made to volume with the acetone-alcohol solution and filtered.<sup>8</sup> A 10 ml. aliquot of the filtrate is evaporated to dryness on the water bath and dissolved in 5.0 ml. of petroleum ether. The solution is placed upon the chromatographic column, containing 2.5 Gm. of aluminum oxide according to Brockman (Merek), and washed with 5.0 ml. of petroleum ether.<sup>12</sup> The ester fraction is eluted by the addition of 50 ml. of 10 per cent ethyl ether in petroleum ether. The eluate is collected and evaporated to dryness on a water bath. The free cholesterol is eluted with 25 ml. of 10 per cent ethyl alcohol in petroleum ether. This eluate is likewise evaporated to dryness.

The cholesterol ester fraction is dissolved in 10 ml. of 95 per cent ethyl alcohol and saponified by heating in an oven at 80 to 90° C. for ten minutes with 0.10 ml. of a potassium hydroxide solution (10 Gm. potassium hydroxide in 20 ml. water). This solution is neutralized with 10 per cent acetic acid in ethyl alcohol, using phenolphthalein as the indicator, one drop more than the required amount of acid is added. The solution is then evaporated to dryness on the water bath.

Both the free cholesterol and the saponified fraction are extracted with anhydrous chloroform and filtered into 10 ml. volumetric flasks. The filtrates are made to volume with chloroform. The reagent for color development is prepared by adding 1.0 ml. of concentrated sulfuric acid to 10 ml. of acetic anhydride. The acetic anhydride is chilled in an ice bath during the addition of the sulfuric acid and is kept there until used. The reagent is prepared fresh for each determination. To 5.0 ml. aliquots of the chloroform solutions is added 1.0 ml. of the reagent, and the color developed is read after standing for ten minutes. A Klett-Summerson colorimeter was used with either filter 54 or 42; the latter was found to be more sensitive.<sup>14, 15</sup> The color is stable for at least ten minutes.

## RESULTS

A standard solution containing 1.0 mg. of cholesterol in 5.0 ml. of petroleum ether was used to obtain a calibration curve. Aliquots of the standard solution were pipetted into colorimeter tubes, evaporated to dryness, and 5.0 ml. of chloroform added to each tube. Color development was carried out as described. The color obtained obeyed Beer's law in the concentration range of 0.02 to 0.50 mg. of cholesterol; above this concentration the color was too dense for accurate reading.

At the same time that the chromatographic separation was performed, another 10 ml. aliquot of the original plasma or serum filtrate was saponified and the total cholesterol determined by the method of Sperry and Brand.<sup>8</sup>

*Accuracy of Separation of Cholesterol From Its Ester.*—Cholesterol stearate was prepared by the method of Page and Rudy.<sup>16</sup> A petroleum ether solution of the ester and cholesterol was prepared containing 1.0 mg. of cholesterol stearate (equivalent to 0.60 mg. cholesterol) and 0.2 mg. of cholesterol in 5.0 milliliters. This ratio represents approximately that found in normal human serum. Twelve separate determinations were made upon aliquots of the solution using the procedure described. The recovery of free cholesterol was  $0.21 \pm 0.01$  mg., and of ester cholesterol,  $0.57 \pm 0.025$  milligram. This represents an accuracy of approximately 5 per cent. It might be noted, however, that the ester recovery generally tended to be about 5 per cent low and the free cholesterol recovery was high by about the same amount. In a series of experiments with free cholesterol alone, a similar plus or minus variation was observed whether

the cholesterol was chromatographed or run directly. This error is probably inherent in the colorimetric method.

*Recovery of Cholesterol Added to Blood Serum.*—Since it was possible to separate cholesterol from cholesterol stearate in pure solution, the separation was next attempted in blood serum. The same serum was analyzed before and after the addition of the ester and cholesterol to the alcohol-acetone solution of the serum extract before filtering. In a 25 ml. flask were placed 15 ml. of the acetone-alcohol solution and 1.0 ml. of petroleum ether containing 0.4 mg. of cholesterol and 1.7 mg. of cholesterol stearate (equivalent to 1.0 mg. of cholesterol), and then 1.0 ml. of serum was added. The procedure as detailed previously was employed. The results of these experiments are given in Table I. Cholesterol can be separated from cholesterol ester in blood serum with the same degree of precision as in pure solution.

TABLE I. RECOVERY OF CHOLESTEROL AND CHOLESTEROL STEARATE ADDED TO BLOOD SERUM

SAMPLE	SERUM				RECOVERY	
	FOUND		FOUND AFTER ADDITION OF 0.40 MG. CHOLESTEROL AND 1.00 MG. CHOLESTEROL AS STEARATE		CHOL. (MG.)	ESTER (MG.)
			CHOL. (MG.)	ESTER (MG.)		
1	0.87	1.05	1.30	2.07	0.43	1.02
2	0.87	1.05	1.25	2.10	0.38	1.05
3	0.85	1.00	1.25	2.06	0.40	1.06
4	0.85	1.00	1.28	1.91	0.43	0.91
Average					0.41	1.01

In order to determine the reproducibility of the results, six separate determinations were made upon the same sample of blood serum. These results are given in Table II. The per cent of free cholesterol found varied from 33.3 to 36.6, with a standard deviation of 0.13 from the mean of 35.1 per cent. There is a deviation of 5 per cent from the mean for the highest and lowest values. The first column gives the data obtained upon the aliquot not chromatographed but analyzed after saponification by the method of Sperry and Brand.<sup>8</sup> In general the agreement between this method and the chromatographic method is good.

*Estimation of Cholesterol and Cholesterol Esters in Blood Serum and Plasma.*—In Table III are presented the data obtained upon ten samples of normal serum, and in Table IV, upon twenty-five samples of blood plasma. The average total amount of cholesterol present as determined by the Sperry-Brand<sup>8</sup> procedure is practically identical with the average total amount obtained by the chromatographic procedure, showing, at least, that there is no loss in the method. There is no significant difference in the ratio of free cholesterol to total cholesterol obtained in the plasma from that in the serum. The ratio is rather constant and is independent of the total amount of cholesterol present. As found by Schoenheimer and Sperry,<sup>3</sup> using digitonin as the precipitant, it is 31.3 expressed as per cent, based upon the data from seventeen subjects. In a subsequent paper Sperry<sup>17</sup> reported a value of  $26.9 \pm 1.4$  per cent based upon data

TABLE II. REPRODUCIBILITY OF ESTIMATIONS OF FREE AND ESTER CHOLESTEROL IN THE SAME BLOOD SERUM

TOTAL* (MG. %)	TOTAL (MG. %)	FREE (MG. %)	ESTER (MG. %)	$\frac{\text{FREE}}{\text{TOTAL}} \times 100$ (%)
158	152	52	100	31.2
158	159	57	102	35.8
151	171	57	111	33.3
152	172	63	109	36.6
157	165	57	108	34.5
152	161	59	105	35.9
Average	151.6	57.5	106.3	35.1 $\pm$ 0.13

\*Method of Sperry and Brand.<sup>4</sup>

TABLE III. CHOLESTEROL AND CHOLESTEROL ESTERS IN NORMAL BLOOD SERUM

	TOTAL*	TOTAL	FREE	ESTER	$\frac{\text{FREE}}{\text{TOTAL}} \times 100$
	(MG. %)	(MG. %)	(MG. %)	(MG. %)	(%)
	138	132	39	93	29.5
	150	137	37	100	27.1
	158	152	52	100	34.2
	144	155	47	108	30.2
	155	156	66	90	42.3
	186	198	83	115	41.9
	186	203	70	133	34.2
	225	229	79	150	34.5
	229	230	75	155	32.6
	240	249	87	162	34.9
Average	181.1 $\pm$ 37.6	184.1 $\pm$ 43.0	63 $\pm$ 18.0	120.6 $\pm$ 27.6	34.1 $\pm$ 4.9

\*Method of Sperry and Brand.<sup>4</sup>

TABLE IV. CHOLESTEROL AND CHOLESTEROL ESTERS IN NORMAL BLOOD PLASMA

	TOTAL* (MG. %)	TOTAL (MG. %)	FREE (MG. %)	ESTER (MG. %)	$\frac{\text{FREE}}{\text{TOTAL}} \times 100$ (%)
	114	112	40	72	35.7
	126	125	50	75	40.0
	136	129	55	74	42.6
	138	132	39	93	29.5
	150	137	37	100	27.0
	136	141	55	86	39.0
	145	143	55	88	38.1
	173	143	42	101	29.4
	150	146	60	86	41.0
	136	150	55	95	36.0
	162	155	62	93	40.0
	144	155	47	108	30.0
	165	170	65	105	38.2
	169	171	58	113	33.9
	172	174	57	117	32.8
	200	175	64	111	36.5
	194	176	66	130	33.6
	175	177	53	124	29.9
	190	184	63	121	34.2
	176	184	67	117	36.4
	190	185	73	112	39.4
	176	186	50	131	25.4
	225	229	88	141	38.4
	236	257	112	145	43.5
	336	349	147	202	42.1
Average	172.6 $\pm$ 45.5	172.6 $\pm$ 49.1	63.6 $\pm$ 24.3	109.0 $\pm$ 28.0	35.8 $\pm$ 4.7

\*Method of Sperry and Brand.<sup>4</sup>

from sixty-two subjects, and he stated that with few exceptions values over 31 per cent were found only in the presence of infection or liver disease.

Using substantially the same method, Sobel and Mayer<sup>18</sup> found the percentage of free cholesterol to total cholesterol to vary from 22.6 to 29.2. On the other hand, Page and co-workers<sup>19</sup> found no such constancy of ratio, but their average value, based on data from sixty-six subjects, was 34.9 per cent free. Other workers<sup>20</sup> used digitonin to precipitate the free cholesterol, and the digitonide was estimated gasometrically. Folch and co-workers<sup>21</sup> have stated that the gasometric values for free cholesterol are 20 per cent too high. Gardner and Gainsborough<sup>22</sup> likewise used digitonin to precipitate free cholesterol which was then estimated colorimetrically and reported 35.3 per cent free cholesterol in twenty-one women and 33.8 per cent in twenty-two men. Recently Foldes and Murphy,<sup>23</sup> using digitonin precipitation, found an average of 33 per cent free cholesterol in the blood plasma of forty normal subjects. Although there appears to be some difference in the percentage of free cholesterol obtained by different laboratories using various methods, it is apparent that there is little disagreement over the fact that the percentage of free cholesterol is quite constant. The chromatographic method gives values for the percentage of free cholesterol within the range of those reported by others.

#### SUMMARY

Cholesterol and cholesterol esters adsorbed upon aluminum oxide from petroleum ether solution can be separated by selective elution. The ester fraction is eluted with 10 per cent ethyl ether dissolved in petroleum ether, and the free cholesterol with a 10 per cent solution of ethyl alcohol in petroleum ether.

The ester fraction is saponified and the free cholesterol in both fractions is determined by the Liebermann-Burchard colorimetric method. The method is rapid, approximately three hours are required for a complete determination, and the results are reproducible with an accuracy within 5 per cent.

The procedure has been applied to normal blood serum and plasma. The per cent of free cholesterol found in ten normal sera averaged  $34.1 \pm 4.9$  per cent, and in twenty-five normal plasmas it averaged  $35.8 \pm 4.7$  per cent of the total cholesterol.

#### REFERENCES

1. Windaus, A. Ueber die quantitative Bestimmung des Cholesterins und der Cholesterinester in einigen normalen und pathologischen Nieren, *Ztschr. f. physiol. Chem.* 65: 110, 1910.
2. Bloor, W. R., and Knudson, W. R. The Separate Determination of Cholesterol and Cholesterol Esters in Small Amounts of Blood, *J. Biol. Chem.* 27: 107, 1916.
3. Schoenheimer, R., and Sperry, W. M.: A Micromethod for the Determination of Free and Combined Cholesterol, *J. Biol. Chem.* 106: 745, 1934.
4. Reinhold, J. G.: The Determination of Blood Cholesterol, Factors Influencing the Accuracy of Various Methods, *Am. J. Clin. Path.* 6: 31, 1936.
5. Yasuda, M.: Contributions to the Microdetermination of Cholesterol, *J. Biol. Chem.* 92: 303, 1931.
6. Noyons, E. C.: Über die kolorimetrische Bestimmung des Cholesterins und seiner Ester mittels die Reaktion nach Liebermann Burchard, *Biochem. Ztschr.* 298: 391, 1938.
7. Kelav, P. P.: Determination of Cholesterol, *J. Biol. Chem.* 127: 15, 1939.

8. Sperry, W. M., and Brand, F. C.: The Colorimetric Determination of Cholesterol, *J. Biol. Chem.* 150: 315, 1943.
9. Trappe, W.: Die Trennung biologischen Fettstoffs aus ihren natürlichen Gemischen durch Anwendung von Adsorptionsäulen, *Biochem. Ztschr.* 307: 97, 1941.
10. Trappe, W.: Eine einfache Methode zur getrennten, quantitativen Bestimmung von freiem und verestertem Cholesterin im Blutserum ohne Digitoninfällung und Verseifung, *Ztschr. f. physiol. Chem.* 273: 177, 1942.
11. Trappe, W.: Eine einfache Methode zur getrennten, quantitativen Bestimmung von freiem und verestertem Cholesterin mit 0.1 ccm. Blut aus der Fingerbeere, *Klin. Wchnschr.* 21: 631, 1942.
12. Veitch, F. P., Jr., and Milone, H. S.: Chromatographic Separation of the 2,4-Dinitrophenylhydrazones of Estrone and Equilenin, *J. Biol. Chem.* 157: 417, 1945.
13. Veitch, F. P., Jr., and Milone, H. S.: Studies on the Sex Hormones; the Quantitative Estimation of Estrone in Pure Solution, *J. Biol. Chem.* 158: 61, 1945.
14. Sunderman, F. W., and Razeq: Spectrophotometric Studies of the Color Development in the Analysis of Sugar by the Benedict Method and of Cholesterol by the Liebermann Burchard Reaction, *J. Biol. Chem.* 118: 397, 1937.
15. Hawk, P. B., Oser, B. L., and Summerson, W. H.: *Practical Physiological Chemistry*, Philadelphia, 1947, The Blakiston Company, p. 538.
16. Page, I. H., and Rudy, H.: Ueber die Fettsäureester des Cholesterins, *Biochem. Ztschr.* 220: 304, 1930.
17. Sperry, W. M.: The Relationship Between Total and Free Cholesterol in Human Blood Serum, *J. Biol. Chem.* 114: 125, 1936.
18. Sobel, A. E., and Mayer, A. N.: Improvements in the Schoenheimer Sperry Method for the Determination of Free Cholesterol, *J. Biol. Chem.* 157: 255, 1945.
19. Page, I. H., Kirk, E., Lewis, W. H., Jr., Thompson, W. R., and Van Slyke, D. D.: Plasma Lipids of Normal Men at Different Ages, *J. Biol. Chem.* 3: 613, 1935.
20. Kirk, E., Page, I. H., and Van Slyke, D. D.: Gasometric Microdetermination of Lipids in Plasma, Blood Cells, and Tissues, *J. Biol. Chem.* 106: 203, 1934.
21. Folch, J., Schneider, H. A., and Van Slyke, D. D.: Notes on Gasometric Plasma Lipid Analyses, *J. Biol. Chem.* 133: 33, 1940.
22. Gardner, J. A., and Gainsborough, H.: Studies on the Cholesterol Content of Normal Human Plasma, *Biochem. J.* 21: 130, 1927.
23. Foldes, F. P., and Murphy, A. J.: Distribution of Cholesterol Esters and Phospholipid Phosphorus in Normal Blood, *Proc. Soc. Exper. Biol. & Med.* 62: 215, 1946.



# RATES OF SODIUM TURNOVER IN NORMAL SUBJECTS AND IN PATIENTS WITH CONGESTIVE HEART FAILURE

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THERE is a need for a study of the rate with which sodium escapes from the blood into the tissue spaces and urine of man, especially because of the present interest in the role of sodium in congestive heart failure and other types of edema. Although studies of sodium metabolism have been conducted in man by means of radiosodium,<sup>1-4</sup> these have not included a detailed tracing of the variations in sodium concentration in the blood, urine, and sweat. The rates of diffusion of sodium through the vascular wall have been determined for other animals<sup>5, 6</sup> but not for man. Furthermore, tracer studies in congestive heart failure with the use of radiosodium have not been conducted previously. These studies are concerned with such problems.

## METHODS

The subjects were all patients at the Charity Hospital. For purposes of discussion, the subjects were divided into three groups: normal subjects, those with congestive heart failure, and miscellaneous subjects. Certain detailed information concerning them is given in Table I. The ten normal subjects were patients who had entered the hospital for observation but were clinically well when studied. The patients with congestive heart failure suffered with chronic congestive heart failure of Functional Class IV and were bedridden. All were under treatment and were improving.

Because of the 14.8-hour half life of the radioactive tracer,  $\text{Na}^{24}$ , and the uncertainty of delivery from the cyclotron laboratories it was not possible to keep patients without treatment in preparation for the experiments. The influence of various therapeutic measures and other details concerning congestive heart failure were evaluated in studies conducted with the use of the long-life sodium,  $\text{Na}^{22}$ .<sup>7, 8</sup> The results of those studies will appear from time to time elsewhere in a series of papers, one report<sup>7</sup> having been published.

All subjects were at least three hours postprandial. They were taken to the laboratory where ureteral catheters were placed‡ into the pelves of each kidney of the normal subjects. The first subjects studied received no drugs in preparation for the cystoscopy while the later ones received morphine

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TABLE I. CLINICAL DATA CONCERNING THE NEGRO SUBJECTS STUDIED\*

GENERAL DATA CONCERNING THE NEGRO SUBJECTS STUDIED*										
SUBJECT	SEX	AGE	WT.	DIAGNOSIS	TREATMENT	Na <sup>24</sup> IN URINE (MEQ./L.)			Na <sup>23</sup> SI TIME OBS. (C.C.)	
						MEAN	MAX.	MIN.	60 MIN.   9	
Normal Subjects										
1	F	37	152	Rheumatoid arthritis	None	123	128	119	12180	
2	F	32	119	Rheumatoid arthritis	None	176	182	179 (lt.)	16598	
3	F	24	94	Pneumonitis	Vitamins	212	219	203 (rt.)	5587	
4	F	17	108	Syphilis	None	116	120	112 (rt.)		
5	F	24	118	Anxiety neurosis	Na salicylates	119	123	115 (lt.)		
6	F	18	110	Syphilis	None	100	103	84	11689	1
7	F	23	146	Normal	None	144	149	139	11786	
8	F	32	100	Normal	None	88	91	85 (lt.)	11534	1
					None	185	192	187	9132	
9	F	19	92	Chronic bronchitis	None	209	216	202 (rt.)	6124	
10	F	34	130	Anxiety neurosis	None	194	201	187 (lt.)		
Mean						216	223	208 (rt.)	10301	11
						209	216	202 (lt.)		
Maximum						263	272	254	8961	
Minimum						163	171	157	10689	11
						157 (lt.)	163 (lt.)	154 (lt.)		
						188 (rt.)	195 (rt.)	181 (rt.)		
						272	254	251	16598	12
						91	84	84	6124	1
Subjects With Congestive Heart Failure										
F	66	121	Arteriosclerotic heart disease, congestive heart failure, syphilis, auricular fibrillation	Digitalis, salt-free diet	62	64	51	12747	165	
F	65	159	Hypertensive cardiovascular disease, congestive heart failure	NH <sub>4</sub> Cl, salt-free diet, digitalis	132	13.7	12.8	12461	135	
F	36	129	Hypertensive cardiovascular disease, congestive heart failure	Salt-free diet, NH <sub>4</sub> Cl, digitalis	162	16.7	15.6	17197	2118	
F	46	125	Hypertensive cardiovascular disease, congestive heart failure	Digitalis, salt-free diet	52	53	50	10315	10220	
F	53	122	Hypertensive cardiovascular disease, congestive heart failure	Digitalis, salt-free diet, Hg (occasionally)	119	123	115	17668	17762	
M	32	137	Hypertensive cardiovascular disease, congestive heart failure	Salt-free diet, NH <sub>4</sub> Cl, digitalis				10163	9259	
F	59	181	Arteriosclerotic heart disease, congestive heart failure	Salt-free diet, NH <sub>4</sub> Cl, digitalis	136	141	132	28624	-----	
F	61	148	Arteriosclerotic heart disease, congestive heart failure		40	41	38	12895	16461	

TABLE 1—CONT'D

SUBJECT	SEX	AGE	WT.	DIAGNOSIS	TREATMENT	Na <sup>23</sup> IN URINE (MEQ./L.)			Na <sup>23</sup> SPACE TIME OBSERVED (C.C.)	
						MEAN	MAX.	MIN.	60 MIN.	90 MIN.
10	F	67	140	Arteriosclerotic heart disease, congestive heart failure	Salt-free diet, digitalis, salyrgan, NH <sub>4</sub> Cl	253	261	239	14674	1673
11	M	62	166	Hypertensive cardiovascular disease, congestive heart failure	NH <sub>4</sub> Cl, digitalis, salt-free diet	100	103	98	9029	997
Mean						87.9	90.7	83.5	14577	1463
Maximum						13.2	26.1	23.9	28624	2118
Minimum						25.3	13.7	12.8	9029	925
<i>Subjects With Miscellaneous Disease States</i>										
1	F	41	151	Hypertensive cardiovascular disease, congestive heart failure, ovarian malignancy	NH <sub>4</sub> Cl, digitoxin salt-free diet	31	32	30	25157	2347
2	F	27	110	Chronic active hemorrhagic nephritis	Low-salt diet				14535	1566
3	F	37	134	Sarcoidosis	Symptomatic	52	53	50	10899	1070
4	F	42	141	Hypertensive cardiovascular disease, pyelonephritis vs. chronic active hemorrhagic nephritis	No diuretic or edema therapy	90	93	87	20833	2061
5	F	50	109	Phlebostrombosis of deep leg veins	No special therapy	25	26	24	10315	1156
6	F	36	173	Cold allergy, anxiety neurosis	None for edema, kidney	132	137	128 (rt.)	10096	115
7	M	15	110	Chronic adhesive pericarditis	NH <sub>4</sub> Cl, salt-free diet, paracentesis	26.5	27.4	25.6		
Mean						44.9	46.3	43.3	15306	1555
Maximum						90	93	87	25157	2347
Minimum						25	26	24	10096	1070

Blood the t were those made at the time of admission to the Charity Hospital for study.

f the sodium (Na<sup>23</sup>) in the urine was determined on the basis of a normal. The "sodium space" was calculated on the basis of a state of equilibrium. The "normal subjects were clinically well when studied; the diagnoses indicated

sulfate, 15 mg., and atropine sulfate, 0.3 mg. subcutaneously thirty minutes before the cystoscopy. The results showed evidence of influence by the drugs used in preparation of the subjects for cystoscopy. The patients with congestive heart failure and two normal subjects had a small catheter passed only into the bladder. The patients with congestive heart failure were too ill to permit a cystoscopy to place catheters into the renal pelvis. Bladder urine was about as informative as the pelvic urine.

After catheterization, a sample of urine was collected from each catheter and a sample of blood was collected simultaneously from the antecubital vein of one arm through a heparinized needle. The radioactive tracer, Na<sup>24</sup> (0.5 to 2 c.c. of a neutral and hypotonic aqueous solution of NaCl), with an activity

of 20,000,000 counts per minute (approximately 0.1 mc. or 0.04 r) was injected quickly into the antecubital vein of the opposite arm. Two cubic centimeter samples of blood were collected for serum studies as rapidly as possible, every five to ten seconds unless the needle became obstructed and necessitated a greater interval between collections. This rate of collection was followed for the first three minutes after the injection of the  $\text{Na}^{24}$ . The interval between collections was increased to thirty seconds for the next five minutes, increased again to every sixty or 120 seconds for the next ten minutes, and finally, to every three, five, or ten minutes for the remainder of the study, which lasted from sixty to one hundred eighty minutes. The urine samples were collected from each catheter in separate vials. For the first three minutes the interval between collections of the urine samples varied with the rate of urine flow, that is, long enough for four drops of urine to be collected. This usually required five to fifteen seconds. For the next five minutes collections were made at thirty-second intervals, etc., as for the blood samples. Attempts were made to collect the blood and urine samples at the same time in order to permit satisfactory comparisons. The volume of the urine samples was measured to determine the sodium ( $\text{Na}^{24}$ ) clearances from collection to collection. The urine samples collected from the pelvis of each kidney were studied separately.

In several experiments samples of edema fluid were collected simultaneously through a puncture wound made through the skin near the ankle with an 18-gauge needle. The rates of collection approximated those for the blood and urine. Simultaneous collections of successive samples of ascitic and pleural fluid in subjects with congestive heart failure were made to study the change in concentration of  $\text{Na}^{24}$ .

One normal subject was placed in a hot and humid room (temperature,  $48^{\circ}\text{C}$ .; relative humidity, 45 per cent) free from drafts. After sweating had become profuse the procedure described above was followed except that the urine was collected at less frequent intervals while the blood and sweat samples were collected at the rapid rates stated previously. The sweat was collected by allowing the beads of sweat to roll into the opening of a tall narrow-mouthed vial which was promptly closed with a bakelite screw cap. Each sample of sweat consisted of about 0.1 cubic centimeter. These collections were continued for about thirty minutes, that is, until the subject and observers began to experience marked discomfort from the hot and humid environment.

The concentration of  $\text{Na}^{24}$  in all samples was expressed as counts per cubic centimeter per minute. Geiger-Muller counters\* were used to determine radioactivities of the injections and the samples. Corrections were made for background counts and decay. The entire counting method had an over-all error of less than 3 per cent. All dosages of  $\text{Na}^{24}$  were reduced to the common value of 20,000,000 counts per minute per 100 pounds of weight.

#### RESULTS

*Normal Subjects.*—The results for the normal subjects are summarized in Figs. 1 to 6.

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*Blood:* It can be seen from Fig. 1 that the concentration of the  $\text{Na}^{24}$  rose rapidly in the *blood serum*, fluctuating in concentration for the first five to fifteen minutes and then becoming relatively constant for the remainder of the period of observation. The concentration of  $\text{Na}^{24}$  did not rise to levels as high as would be expected if all the  $\text{Na}^{24}$  remained in the blood stream. There was a tendency for the concentration of  $\text{Na}^{24}$  in the serum of most of the subjects to rise for the first ten to twenty minutes to levels higher than those reached during the final periods of the study and to show marked variations from subject to subject. There was a gradual decline in the serum concentration of  $\text{Na}^{24}$  in all subjects throughout the entire period of study even after a fairly stable state had been reached. The  $\text{Na}^{24}$  did not appear in the antecubital vein of the sampling arm until nineteen to twenty-three seconds after the radiosodium had been injected into the antecubital vein of the other arm, that is, by a time equal to the circulation time.

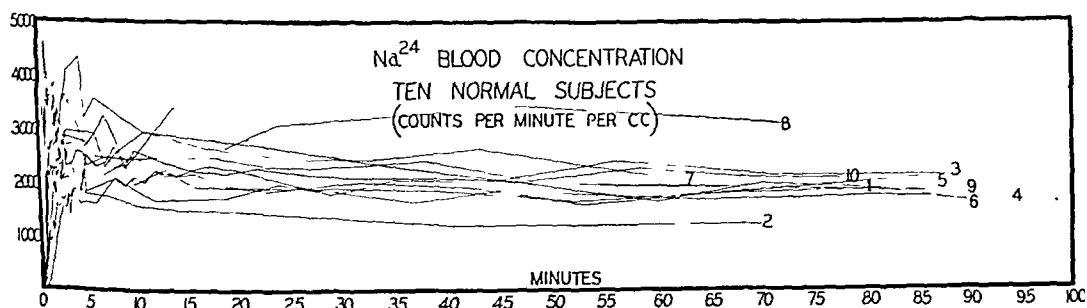


Fig. 1.—Variations in serum concentration of  $\text{Na}^{24}$  in ten normal subjects. There are considerable individual variations, especially during the first two to three minutes when the mixing of the injected radiosodium is occurring.

Because of the marked variations in concentration of  $\text{Na}^{24}$  in the serum of the subjects and because of the small number of subjects studied, it was impossible to obtain a composite tracing which properly depicted the rate of diffusion of sodium from the vascular bed (Fig. 1). Therefore, the data on a subject (Subject 6) were selected for analysis in the manner described by Gellhorn and co-workers;<sup>5</sup> all diffusion curves were drawn by inspection. These authors showed that the vascular bed of dogs could be considered to be composed of two separate areas through which the sodium ion diffuses at different rates. Area  $A_1$  consists of an area through which the sodium diffuses at a very rapid rate, while  $A_2$  represents another area through which the rate of diffusion is relatively slow. The same types of areas were observed in these studies in the normal and diseased man. Fig. 2 can be seen to represent a typical double exponential curve for the blood concentration. There was a rapid rate of escape of  $\text{Na}^{24}$  from the vascular bed and a slow one. The general expression obtained by Gellhorn and co-workers<sup>5</sup> for dogs, and found to apply to man in many of the subjects of this study, is

$$c_p - c_{eq} = a_1 e^{-b_1 t} + a_2 e^{-b_2 t} \quad (1)$$

where  $c_p$  is plasma concentration of  $\text{Na}^{24}$  at time,  $t$ ,  $c_{eq}$  is plasma concentra-

tion at equilibrium,  $b_1$  and  $b_2$  are rates of diffusion from the vascular bed, and  $a_1$  and  $a_2$  are coefficients which depend upon the rates of movement and distribution of  $\text{Na}^{23}$  through areas  $A_1$  and  $A_2$  in the vascular bed. Since the diffusion of  $\text{Na}^{24}$  is determined by the diffusion of  $\text{Na}^{23}$ , the exponents  $b_1$  and  $b_2$  are expressions of the rates of diffusion of the total plasma sodium ( $\text{Na}^{23}$ ) out of the vascular bed. Therefore, it is possible to consider the sodium of the plasma as being divided into three parts: one represented by  $a_1$ , which diffuses outward rapidly through area  $A_1$ ; a second represented by  $a_2$ , which diffuses outward at a relatively slow rate through area  $A_2$ ; and a third represented by  $c_{eq}$ , which remains in the plasma.

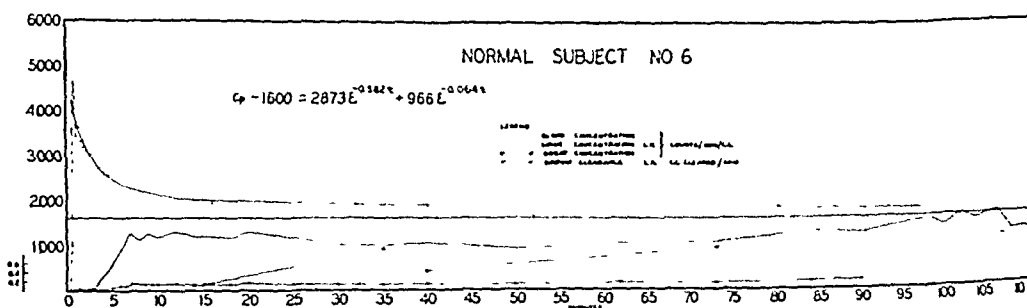


Fig. 2.—Variations in serum, urine, and sweat concentrations of  $\text{Na}^{24}$  and the  $\text{Na}^{24}$  clearance in normal Subject 6. The curve of blood concentration is a double exponential curve representing sodium diffusion, the equation of which is shown. L.K., Left kidney.

To determine the rate of loss by diffusion of the total plasma sodium, the equation

$$\frac{-a_1b_1 - a_2b_2}{c_0} = R_p$$

is employed, where  $c_0$  is the extrapolated initial count per minute per cubic centimeter of the plasma.

The expression for radioactive sodium concentration in the plasma for Subject 6 (normal) was

$$c_p - 1600 = 2873e^{-0.582t} + 966e^{-0.064t} \quad (2)$$

Thus 58 per cent of the rapidly diffusing sodium left the plasma per minute, and 6.4 per cent of the slowly diffusing sodium left the plasma per minute. Considering the sizes of these sodium compartments in the plasma, an average of 32 per cent of the total sodium in the plasma diffused out of the vascular bed per minute.

Furthermore, Merrell and associates<sup>6</sup> have shown that from the same data the rate of diffusion of sodium from the extravascular fluid\* back into the plasma at equilibrium could be determined by the equation

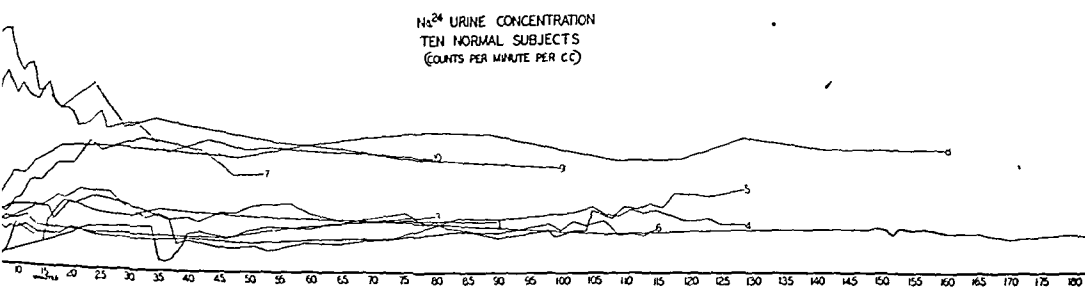
$$R_0 = R_p \frac{(1-q)}{q}$$

\*The term extravascular sodium employed here probably is concerned predominantly with interstitial fluid sodium because of the relatively short duration of the studies. For that reason interstitial and extravascular sodium is considered essentially synonymous in these studies.

where  $R_e$  = rate at which the extravascular sodium returns by diffusion into the plasma,  $R_p$  = rate of loss by diffusion of the total plasma sodium in the vascular bed, and  $q$  = total extravascular sodium. The values obtained for Subject 6 are shown in Table II. It is noted that about 32 per cent of the total sodium in the plasma diffused into the interstitial fluid per minute and that 13 per cent of the total sodium in the interstitial fluid returned by diffusion to the plasma per minute.

TABLE II. PROPORTION OF SODIUM DIFFUSION IN FOUR HUMAN SUBJECTS

	NORMAL SUBJECT 6	CONGESTIVE HEART FAILURE SUBJECT 3	1	MISCELLANEOUS SUBJECT 5
Proportion of total sodium in interstitial fluid	0.70	0.89	0.90	0.65
Proportion of total plasma sodium diffusing out of vascular bed per minute	0.32	0.63	0.54	0.35
Proportion of total interstitial fluid sodium diffusing into the vascular bed per minute	0.13	0.08	0.06	0.18


 Fig. 3.—Variations in urine concentrations of  $\text{Na}^{24}$  in ten normal subjects.

*Urine:* The concentration of radioactive sodium in the urine exceeded that in the blood serum in three subjects (Fig. 3). In most cases the blood and urine concentrations were essentially equal, although the urine concentration was definitely lower in Subject 6. The urine concentration varied much more than did the blood concentration after the initial few minutes following the injection.

*Sodium Clearance:* The sodium clearances for five normal subjects are shown in Figs. 4, 5, and 6.  $\text{Na}^{24}$  clearances were employed in these analyses since they integrated volume of urine flow and blood and urine concentrations. The exact significance of these values for a substance like sodium is not known. They do make certain comparisons feasible. These values were calculated in a manner similar to that used for the urea clearance.

The equation for the calculation is

$$\text{Na}^{24} \text{ clearance} = \frac{\text{Na}^{24} U_v}{\text{Na}_b^{24}} \quad (3)$$

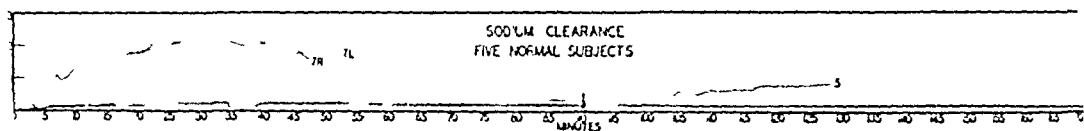


Fig. 4.—Variations in  $\text{Na}^{24}$  clearance in five normal subjects. Since the movement of  $\text{Na}^{24}$  around the body is determined by the movement of  $\text{Na}^+$ , these curves represent rates of  $\text{Na}^{24}$  clearance once equilibrium has been reached.

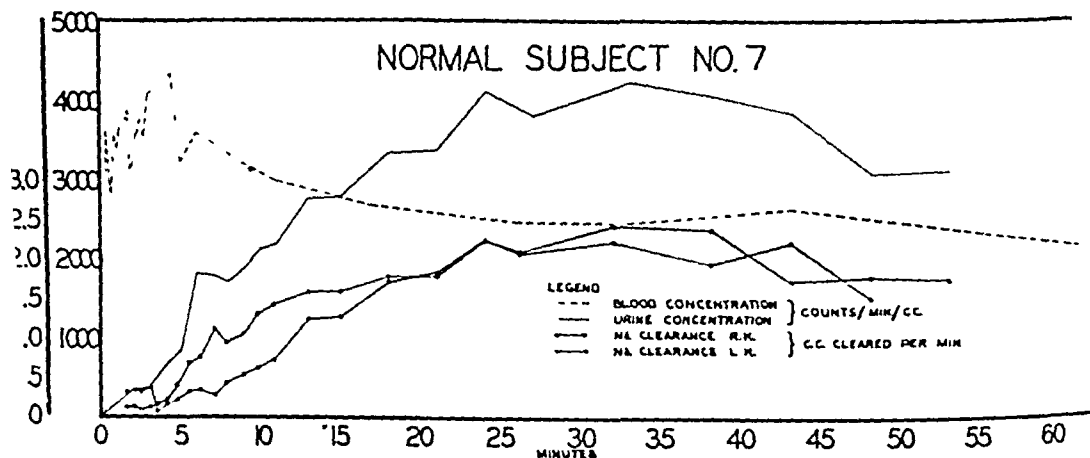


Fig. 5.—Variations in serum and urine concentration (left kidney) of  $\text{Na}^{24}$ , and  $\text{Na}^+$  clearance for both kidneys separately in one subject. Note the marked variations in serum concentration during the first three minutes. The serum concentration then settled down to a diffusion type of curve as shown for normal Subject 6 (Fig. 2). The mixing phenomenon was relatively poor for the first three minutes in Subject 7, but early and adequate for Subject 6. Such chance variations are to be expected. Note the concordant and only occasional discordant relationship of the  $\text{Na}^{24}$  clearances in the urines of the right and left kidneys.

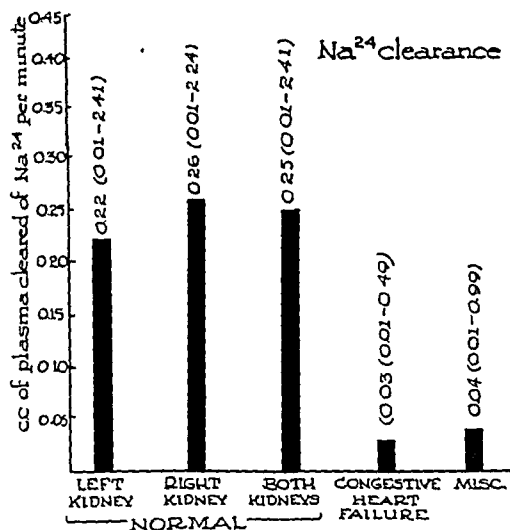


Fig. 6.—The average and extreme (within parentheses) values for  $\text{Na}^{24}$  clearance in the three groups of subjects. It is necessary to remember that the patients with congestive heart failure were on a slow sodium diet.



where  $\text{Na}_u^{24}$  = counts per cubic centimeter of urine,  $\text{Na}_b^{24}$  = counts per cubic centimeter of blood, and  $U_v$  = volume of urine flow per minute.

Except for Subject 7 the rates of  $\text{Na}^{24}$  clearance were fairly constant and of essentially the same magnitude. They tended to average 0.22 c.c. of blood cleared per minute per kidney but were high for Subject 7, reaching a level of about 2.4 c.c. of blood cleared of  $\text{Na}^{24}$  per minute for both kidneys. Figs. 4, 5, and 6 show the quantitative and qualitative variations in the clearances for both kidneys observed separately and simultaneously.

*Comment:* The levels of  $\text{Na}^{24}$  concentration in the blood serum should have reached values of about 10,000 counts per minute per cubic centimeter if all of the injected materials had remained in the blood. Instead, the concentrations actually reached averaged about 2,500 counts per minute per cubic centimeter, with the maximum being 4,600. The average curve for the normal subject shows a tendency for the concentration of  $\text{Na}^{24}$  in the blood to reach the final concentration level rapidly and not to exceed it appreciably. This indicates the very rapid rate of diffusion of sodium from the blood. In fact, the diffusion is so rapid that the circulation of the blood is too slow to permit adequate mixing before a considerable amount of diffusion occurs. The relatively marked variations in  $\text{Na}^{24}$  concentration in the blood observed during the first few minutes of the study are due in a large part to inadequate mixing. Adequate mixing does not appear to be completed in some subjects before ten minutes have elapsed (Fig. 1). As stated previously, the marked variations in the rates of mixing of the blood made it impossible to determine the average diffusion rates for the normal subjects as a group. From the analysis of the data of Subject 6 it was found that sodium diffused across the vascular bed at two separate rates. This diffusion outward resulted in a loss per minute of about 0.6 of the rapidly diffusing and of about 0.06 of the slowly diffusing plasma sodium. The average rate of diffusion of sodium from the vascular bed was 32 per cent of the total plasma sodium per minute. This is equal to about 4,000 Gm. of sodium per day for a man weighing 70 kilograms. These results are remarkably similar to those observed in dogs by Gellhorn and associates.<sup>5</sup> It is interesting to note that if the Cl ion tends to follow the Na ion during its diffusion, this would entail a diffusion of about 10,000 Gm. of NaCl out of the vascular bed of the average man per day. The amount of sodium returning is, of course, the same. Thus there is a diffusion of about fifty pounds of NaCl back and forth through the capillary wall per day in a man weighing 70 kilograms.

It is generally agreed that the rapid diffusion of sodium is into the interstitial fluid.<sup>5</sup> The slower diffusion must take place into bone,<sup>2, 9</sup> cerebrospinal fluid,<sup>10</sup> chambers of the eye,<sup>10</sup> and other structures of the body which allow relatively slow rates of sodium exchange. It is also important to know the rate of diffusion of water across the vascular bed. Merrell and co-workers<sup>6</sup> observed that 1.46 of the plasma water diffused out into the tissues of the guinea pig per minute. Since comparable values were obtained for the rates of diffusion of sodium in guinea pigs, dogs, and man, it is likely that the rates of diffusion of water should be similar. If the same rates are applied for man as were

found in the guinea pig, then about 6,300 liters of water per day diffuses out of the vascular bed of a man weighing 70 kilograms. It is obvious from the diffusion rates for sodium and water that water diffuses more rapidly. Not only has this been observed for the body as a whole, but for isolated tissues as well. Kinsey and associates<sup>10, 11</sup> found this to be true for the vitreous body and aqueous humor of the eye of the rabbit. It has also been noted for the cerebrospinal fluid.<sup>12</sup>

Although the  $\text{Na}^{24}$  concentration in the blood serum reached a level free from marked fluctuations within a few minutes, the concentrations progressively decreased and did not reach a state of equilibrium by the end of two hours, even though corrections were made for loss of  $\text{Na}^{24}$  in the urine. These findings, therefore, indicate that the  $\text{Na}^{24}$  rapidly reaches equilibrium with the interstitial fluid. This represents the greatest portion of the  $\text{Na}^{24}$  injected. Then for several hours there is a gradual passage of the  $\text{Na}^{24}$  into bone, spinal fluid, vitreous body, aqueous humor, and similar sodium compartments.<sup>9-11</sup> Therefore, studies which require a complete state of equilibrium with radio-sodium for all structures of the body must be conducted several hours after the injection of the tracer. The measurement of the sodium compartment of the body cannot be measured properly until the tracer has reached a state of equilibrium for all structures of the body. For that reason any estimate of the sodium compartment with the use of  $\text{Na}^{24}$  in the studies reported here is subject to error and therefore has limited significance.

The urine concentration of  $\text{Na}^{24}$  varied considerably from subject to subject. These differences must be related, in part, to the sodium intake preceding the experiments. The cause of the delay in the appearance of the  $\text{Na}^{24}$  in the urine is unknown. It must be due partly to the time required for the urine to flow from the glomerulus to the outlet of the catheter. However, this requires only about thirty seconds. The delay might be due in part to diffusion from the blood into Bowman's capsule, but the rapid appearance of  $\text{Na}^{24}$  in the sweat and the rapid diffusion into the interstitial fluid tends to eliminate such a delay as an important factor. The effect of pooling of urine in the pelvis of the kidneys could not be evaluated.

Although the data are not shown for both kidneys separately, for all subjects the rates of excretion of  $\text{Na}^{24}$  were essentially the same for both kidneys. Although there were periods when the rates of excretion and  $\text{Na}^{24}$  concentration showed discordant relationships, the behavior was so similar as to make it unnecessary to report the data for both kidneys separately. Subject 7 (Fig. 5) exemplified the individual kidney relationships. Since the  $\text{Na}^{24}$  clearances are reported for only one kidney, the total clearances are essentially twice that shown for one kidney.

Although the concentration of  $\text{Na}^{24}$  in the urine varied quite markedly (Fig. 3), the rate of  $\text{Na}^{24}$  clearance was fairly constant. This indicates that when the volume of urine flow was great, the concentration of  $\text{Na}^{24}$  in the urine was proportionately low and vice versa.

*Subjects With Congestive Heart Failure.*—The results obtained in the subjects with congestive heart failure are summarized in Figs. 6 to 11.

**Blood:** There was a tendency for the concentration of  $\text{Na}^{24}$  in the blood serum to reach a higher level in the subjects with congestive heart failure than in the normal subjects (Fig. 7). Marked fluctuations were noted for the first ten to twenty minutes; then a stable level was reached. The injected  $\text{Na}^{24}$

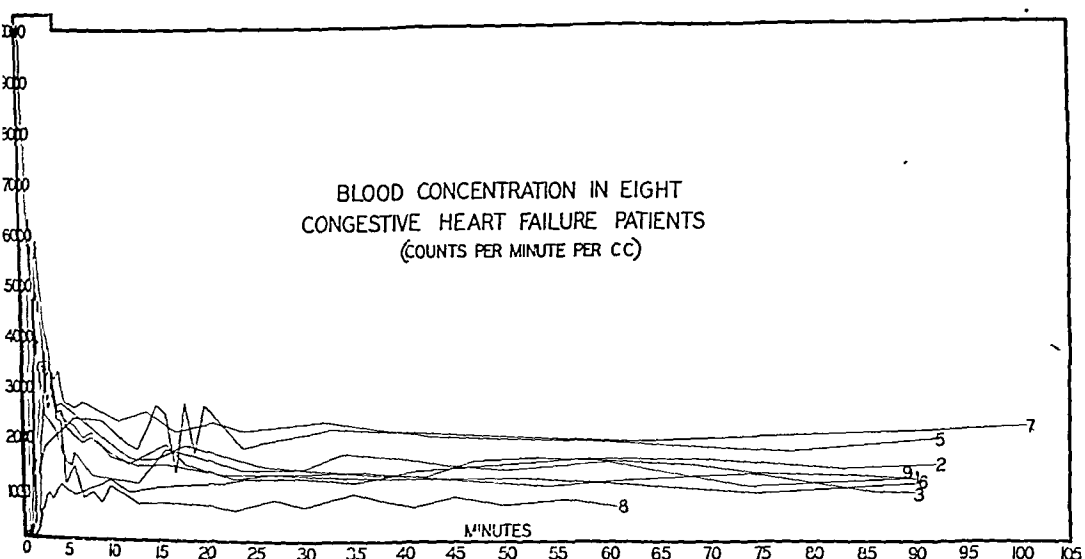


Fig. 7.—Variations in serum concentration of  $\text{Na}^{24}$  in eight patients with congestive heart failure. If the presence of a diffusion type of sodium curve indicates adequate and early mixing of the injected radiosodium, then mixing appears to have developed more quickly in these persons than in the normal ones.

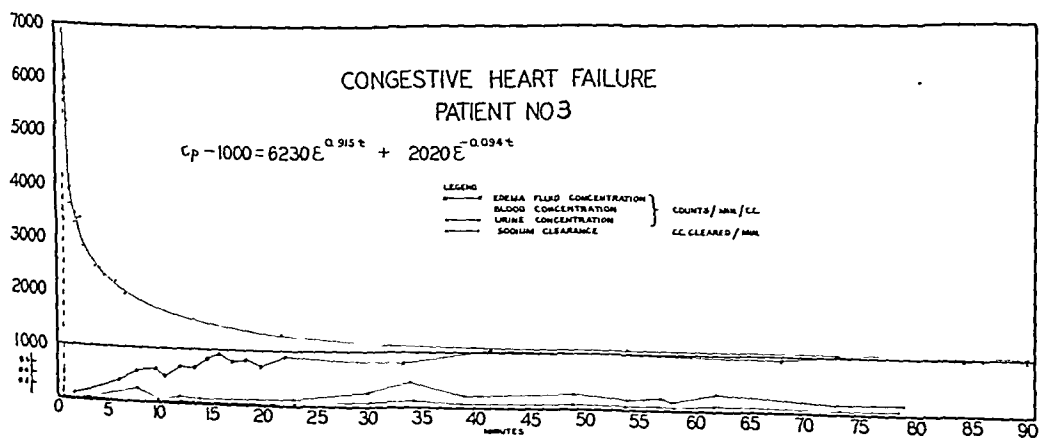


Fig. 8.—Variations in serum, urine, and edema fluid concentration and  $\text{Na}^{24}$  clearance in Patient 3 with chronic congestive heart failure. The serum concentration curve is a typical double exponential diffusion curve, the equation of which is shown. Note the fairly rapid increase in concentration of  $\text{Na}^{24}$  in the edema fluid to a level equal to that in the serum.

appeared in the antecubital vein of the sampling arm a few seconds later than in the normal subjects. Like the normal subjects, the patients with congestive heart failure showed such wide variations in the serum concentrations of  $\text{Na}^{24}$  that it was impossible to calculate a satisfactory average diffusion pattern for the  $\text{Na}^{24}$ . Some individuals, however, presented typical diffusion curves with

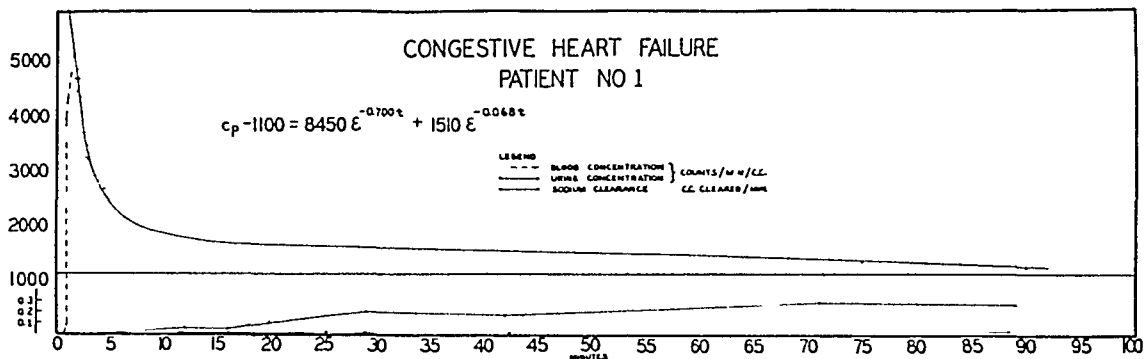


Fig. 9—Variations in serum and urine concentration of  $\text{Na}^+$  and  $\text{Na}^+$  clearance in Subject 1 with congestive heart failure. The equation for the sodium diffusion curve is shown.

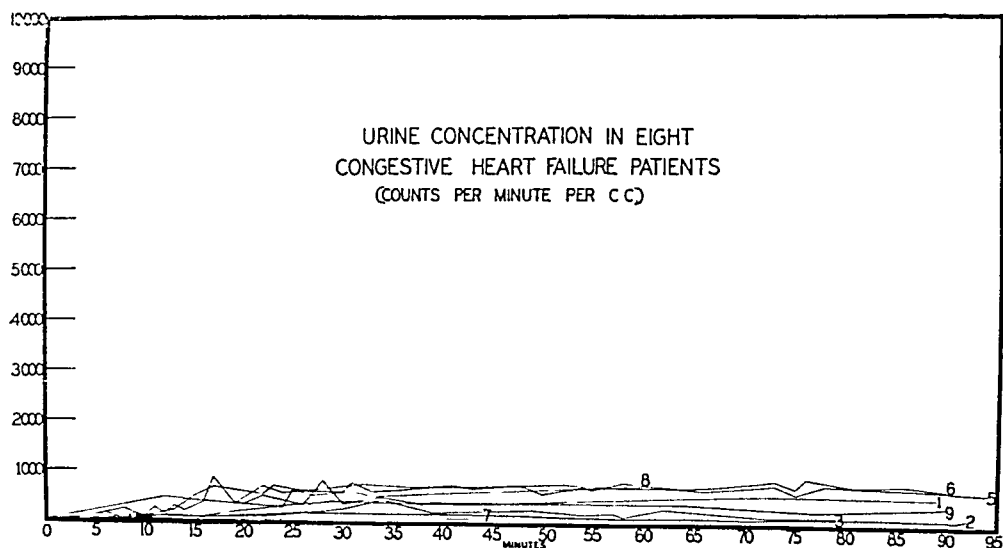


Fig. 10—Variations in urine concentration of  $\text{Na}^+$  in eight patients with congestive heart failure and a low sodium diet. Compare these values with those for the normal (Fig. 3).

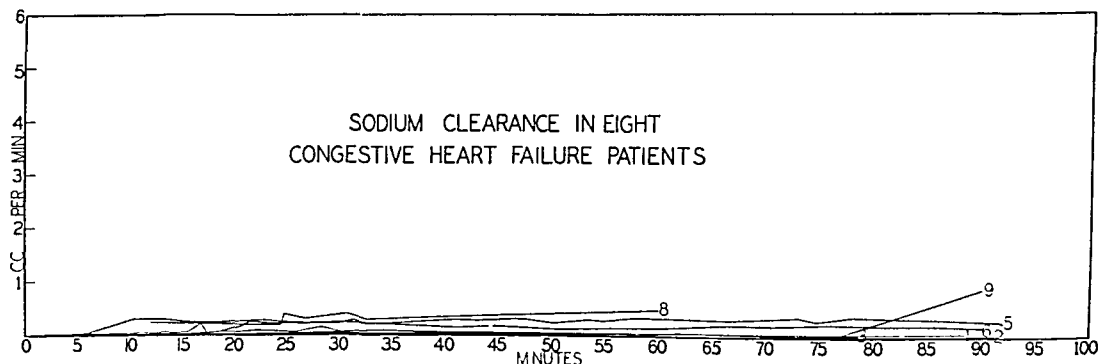


Fig. 11—Variations in  $\text{Na}^+$  clearance in eight patients with congestive heart failure and a low sodium diet. Compare these values with those for the normal (Figs. 4 and 6).

both rapid and slow rates of diffusion of sodium from the blood plasma (Fig. 8). The equation for the diffusion curve of Fig. 8 was found to be:

$$c_p - 1000 = 6230e^{-0.915 t} + 2015e^{-0.094 t} \quad (4)$$

This indicates that 0.915 of the plasma sodium which diffuses at the rapid rate escaped from the vascular bed into the interstitial spaces per minute, and 0.094 of the plasma sodium which diffuses at the slow rate escaped from the vascular bed per minute. Because of the rapid rate of diffusion in Subject 3, another patient (Subject 1) with congestive heart failure was similarly studied (Fig. 9). The curve for this patient had the following equation:

$$c_p - 1100 = 8450e^{-0.700 t} + 1510e^{-0.068 t} \quad (5)$$

The diffusion rates in this subject were about the same as for the normal subject, although still more rapid.

From Table II it can be seen that 54 and 63 per cent of the total plasma sodium diffused out of the vascular bed per minute as average rates in Subjects 1 and 3, respectively. Simultaneously 6 and 8 per cent of the total interstitial fluid sodium diffused back into the plasma per minute in the respective patients.

*Urine:* The urine was collected from the bladder and not directly from the renal pelves as in the normal subjects. The results are summarized by Figs. 8, 9, and 10. The  $\text{Na}^{24}$  did not appear in the urine until four to five minutes after injection except in one patient (Fig. 10). The concentration of  $\text{Na}^{24}$  rose more slowly and the concentration levels reached were much lower than in the normal subjects. Considerable fluctuations were observed during the initial twenty to thirty minutes of study, followed by a stable level with minor variations of essentially the same degree as those encountered in the normal subjects.

*Sodium Clearance:* The sodium clearances of the patients with congestive heart failure with therapy are summarized in Figs. 6, 8, 9, and 11. The rates by which the blood was cleared of sodium were considerably lower than those observed for the normal subjects. These clearance values for both kidneys were about 0.03 c.c. per minute, the quantitative and qualitative variations being shown in Fig. 11. The clearance values approached zero in some subjects (Subjects 1, 2, and 3).

*Edema Fluid:* It was possible to follow the appearance of  $\text{Na}^{24}$  in the edema fluid of most of the patients with congestive heart failure. The results of the study of the edema fluid of Subject 3 are shown in Fig. 8.

*Comment:* The rapid and slow rates of diffusion of sodium from the vascular bed of the patients with congestive heart failure were higher than those observed in the normal subject. Obviously, it is not possible to conclude that such a difference is characteristic of congestive heart failure. Too few subjects were studied and too many factors are concerned with the escape of the sodium ion into the tissues for one to conclude that a greater rate of transcapillary diffusion is at work in all patients with congestive heart failure. The problem of mixing of the blood and the general inefficiency of the circula-

tion are important in congestive heart failure. Furthermore, it is necessary to bear in mind that all of the patients with congestive heart failure were receiving therapy. One aspect of the therapy (ammonium chloride and mercurial diuretics) was intended to make the kidneys excrete more sodium, while another phase of the therapy (dietary restriction of sodium) resulted in a decreased rate of renal excretion of sodium. The influence of these factors on the data obtained is unknown, but they were most probably great enough to make it impossible to evaluate the role of uncomplicated congestive heart failure on transepillary diffusion of sodium. Cellhorn and associates<sup>5</sup> found the rates of transepillary diffusion in dogs in shock, with and without treatment, were 50 per cent of those of normal dogs. Of course, the disturbances in hemodynamics are quite different in shock and in chronic congestive heart failure without shock.

Nevertheless, the more rapid rate of diffusion of sodium across the vascular wall in congestive heart failure than in the normal condition can be explained by the fact that there is a greater quantity of total amount of extracellular fluid and, therefore, of sodium in the edematous patients. Inspection of Table II shows that the part of the total extracellular fluid sodium of the patient with congestive failure diffusing back into the vascular bed per minute is about 50 per cent of that of the normal. The total quantity of sodium concerned, however, is greater than normal. Since the same quantity of sodium returns to the circulation as leaves it per unit time and since the increase in blood volume is not proportional to the increase in volume of the interstitial fluid, it is necessary that the plasma sodium diffuse more rapidly than in the normal subject in order to maintain a state of equilibrium. Thus the sodium of the interstitial fluid of the normal subject is turned over twice as rapidly as in the patients with congestive heart failure, whereas the sodium of the plasma of the latter patients is turned over twice as rapidly as that of the normal.

The rise in the concentration of  $\text{Na}^{24}$  in the edema fluid (Fig. 8) is an index of the rate of diffusion of normal sodium,  $\text{Na}^{23}$ , into edema fluid from the vascular bed. This rate is relatively high when the quantity of the edema fluid is considered. The concentration of the  $\text{Na}^{24}$  in the edema fluid reached that in the plasma, remaining near the plasma level thereafter. The duration of the rising periods of the  $\text{Na}^{24}$  concentration in the edema fluid is further support of the relatively long period of time required for a state of sodium equilibrium to be reached when tracer studies are done. This is particularly true when certain abnormal states are under observation.

#### *Miscellaneous Subjects.—*

*Blood and Urine Concentrations and Sodium Clearance:* The results are summarized in Figs. 12 to 15. The nature of the variations in  $\text{Na}^{24}$  concentration in the blood and urine and  $\text{Na}^{24}$  clearances was essentially the same as for the normal subjects and those with congestive heart failure, differences being determined by the underlying disease state. For example, Subject 1 suffered with congestive failure due to essential hypertension and was under treatment

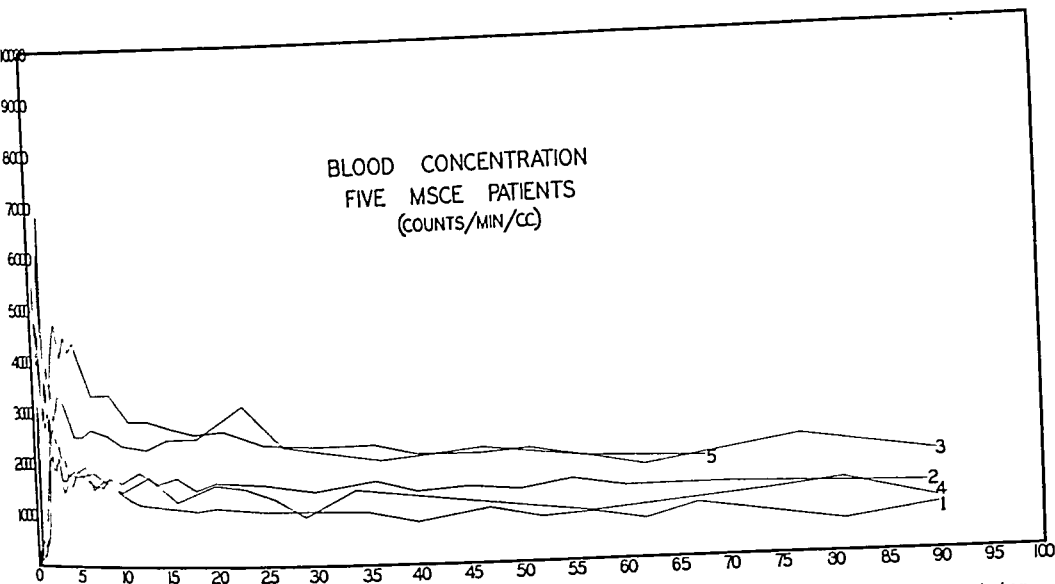


Fig. 12.—Variations in serum concentration in five patients with miscellaneous disease states (Table I).

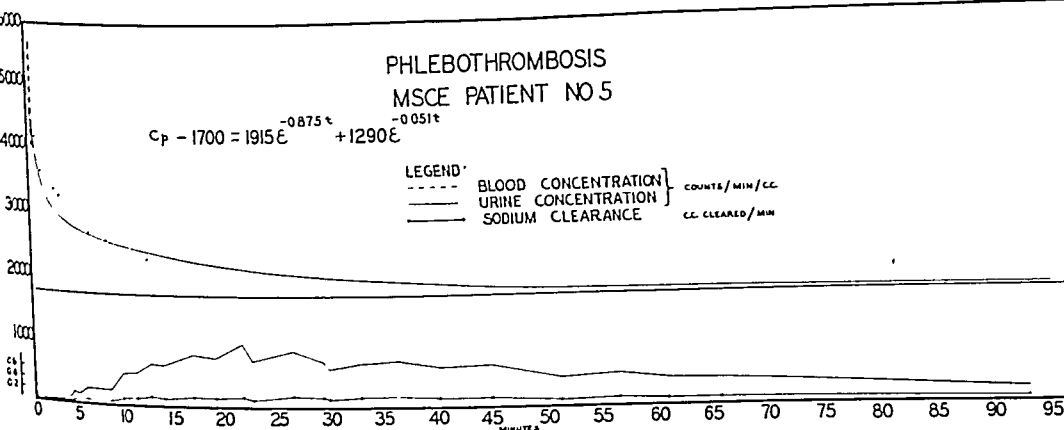


Fig. 13.—Variations in serum concentration, urine concentration, and  $\text{Na}^{24}$  clearance in a patient with chronic phlebothrombosis of both legs and moderate chronic edema. The diffusion curve for sodium and its equation are shown.

for the heart failure. She also had a fairly extensive ovarian malignancy. The variations in  $\text{Na}^{24}$  concentration in the blood and urine and in the sodium clearances were similar to those described for the group of patients with uncomplicated congestive heart failure under treatment. The patients with phlebothrombosis and sarcoidosis were not found to show any special characteristic behavior.

Subject 5 with phlebothrombosis was selected for analysis of the diffusion rates of sodium because the edema was limited to the two lower extremities and because the data were suitable for such an analysis. The curve (Fig. 13) representing variations in the serum concentration of  $\text{Na}^{24}$  is similar to that

of the normal subject and the subjects with congestive heart failure. The curve of the serum concentration may be expressed as

$$c_p - 1700 = 1915e^{-0.875 t} + 1290e^{-0.051 t}, \quad (6)$$

that is, 0.875 of the plasma sodium which diffuses through area  $A_1$  left the blood per minute, while 0.051 of the plasma sodium which diffuses through  $A_2$  left the blood per minute.

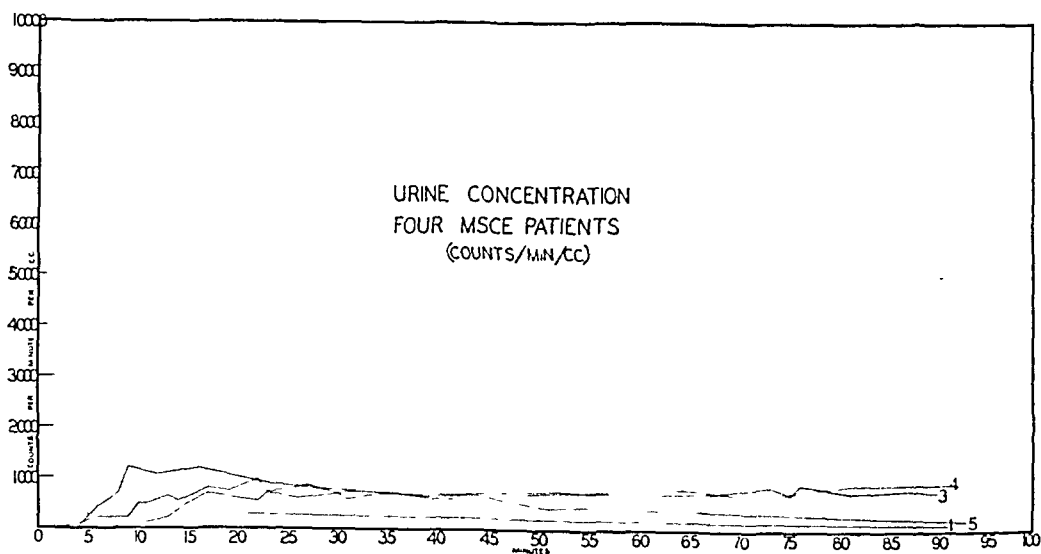


Fig. 11.—Variations in urine concentration of  $\text{Na}^{24}$  in four patients with miscellaneous disease states.

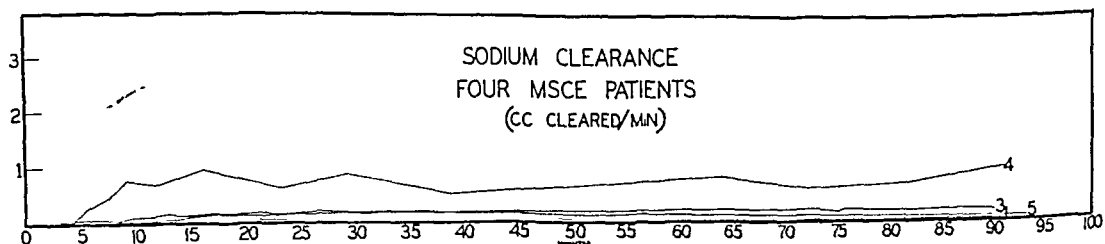


Fig. 15.—Variations in  $\text{Na}^{24}$  clearance in four patients with miscellaneous disease states. \* Compare these values with those of the other two groups of subjects (Figs. 4, 6, and 11).

*Comment:* Fever and other systemic influences from infection, malignancy, or sarcoidosis did not present any detectable influences on the diffusion of sodium through the vascular bed. The rate of excretion through the kidneys, however, might conceivably be influenced by renal damage as well as by therapy. The rates of sodium diffusion were too high to permit minor variations to manifest themselves sufficiently for detection.

The transepillary diffusion of sodium in these subjects indicated at least two rates, a rapid and a slow one. In the subject with phlebothrombosis the rates of diffusion through areas  $A_1$  and  $A_2$  were of the order encountered in the two patients with congestive heart failure. The patient with phlebothrombosis



was receiving only bed rest; no therapy was directed at the edema specifically. Table II indicates the proportion of plasma and interstitial sodium diffusing through the vascular bed per minute.

*Concretio Cordis and Marked Ascites:* Miscellaneous Subject 7 had marked chronic cardiac tamponade with marked ascites and was receiving therapy for congestive heart failure. The therapy included the use of digitalis, mercurial diuretics, and a low sodium diet. He died at operation several days after this study and a thick fibrous sheet, which interfered markedly with diastolic filling of the heart, was found to encase the heart. He was studied in the manner previously described except that an 18-gauge needle was placed into the peritoneum so that samples of ascitic fluid could be collected at frequent intervals for study. The relative concentrations of  $\text{Na}^{24}$  in the blood and ascitic fluid are shown in Fig. 16. There was a very slow but definite rise in the  $\text{Na}^{24}$  concentration in the ascitic fluid. This rise was much slower than that observed in the urine or edema fluid of the other patients. The blood serum concentration of  $\text{Na}^{24}$  showed the usual variations.

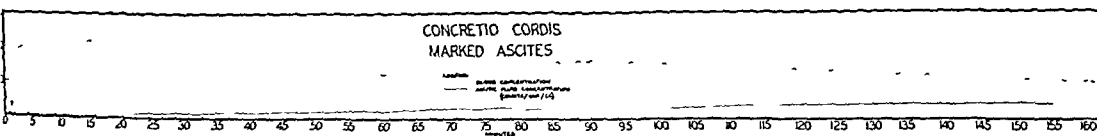


Fig. 16.—Variations in serum and ascitic fluid concentrations of  $\text{Na}^{24}$  in a patient with concretio cordis and marked ascites. Note the relatively slow increase in the  $\text{Na}^{24}$  concentration in the ascitic fluid as compared with that of the edema fluid of Subject 3 with congestive heart failure.

*Comment:* The relatively slow diffusion of  $\text{Na}^{24}$  into the ascitic fluid of this patient is probably due to the great mass of fluid through which the  $\text{Na}^{24}$  must diffuse, as well as a less rapid diffusion of sodium through the peritoneal membrane. The importance of the former factor is supported by the slow diffusion into the edema fluid. The tissues are richly supplied with capillaries which permit a larger area of contact with the interstitial fluid; that is, the ratio of area of capillary wall in contact with each unit volume of fluid is high. The capillary area of contact per unit volume of edema fluid is less and, therefore, a longer time is required for equilibrium to be established. In the case of the ascitic fluid the ratio of capillary wall per unit volume of fluid into which diffusion occurs is considerably less and, therefore, the slower diffusion and even longer time required for equilibrium to be established becomes evident. There is no doubt that the stirring of the edema fluid that occurs with breathing and movement of the body aids in increasing the rate with which the state of equilibrium is reached. These observations further support the evidence that several hours are required to reach equilibrium, especially in edema states, after radiosodium tracer studies are conducted.

*Excretion of  $\text{Na}^{24}$  by the Sweat Glands:* A normal subject (a 45-year-old white man) rested in a hot and humid air-conditioned room (temperature,  $48^{\circ}\text{C}$ .; relative humidity, 45 per cent). After the bladder was emptied he was studied in the same manner as described for the other subjects.

*Results:* The results are summarized in Fig. 17. The  $\text{Na}^{24}$  appeared in the sweat in seventy-five seconds after the intravenous injection. The concentration of  $\text{Na}^{24}$  was greatest in the blood and least in the urine. The concentration in the sweat tended to follow that in the blood very closely. Unfortunately the subject could not remain very long in the hot and humid atmosphere; therefore, the experiment was stopped after only thirty minutes of observation. Another sample of urine and of blood, collected about forty minutes, after the subject was removed from the hot room, showed no special change in  $\text{Na}^{24}$  concentration. Concentrations of  $\text{Na}^{24}$  in the urine, blood, and sweat of normal Subject 6 are shown in Fig. 2. Six samples of sweat were measured during the period of study.

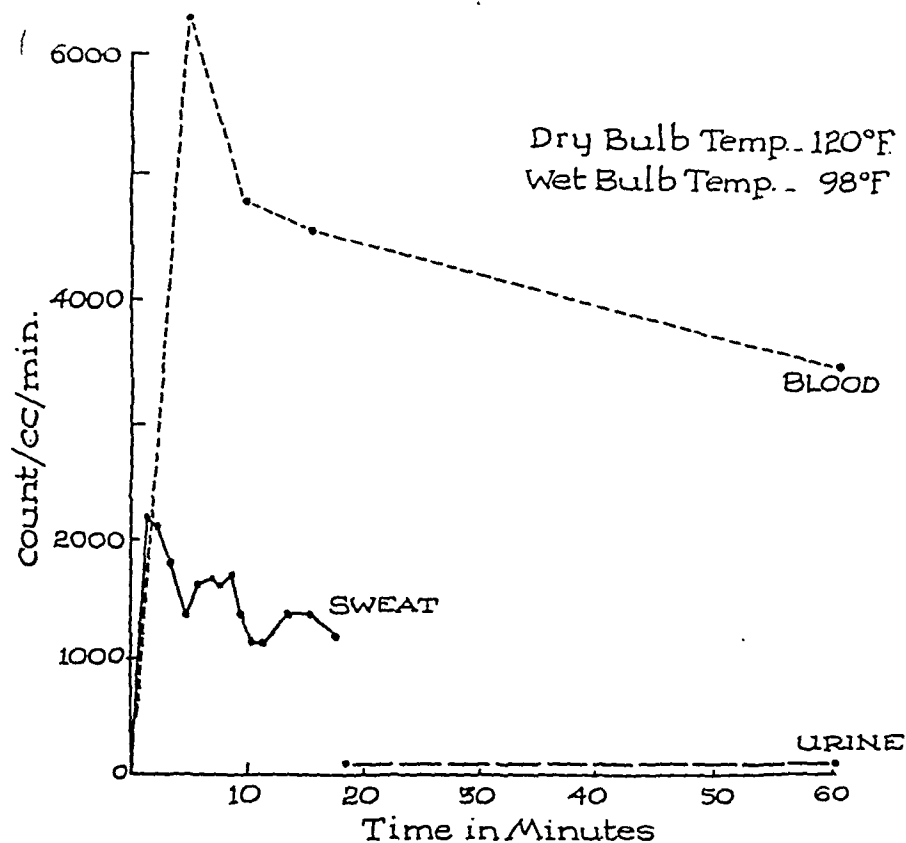


Fig. 17.—Variations in serum, sweat, and urine concentrations of  $\text{Na}^{24}$  in a normal subject who was in the process of profuse thermal sweating when the  $\text{Na}^{24}$  was injected intravenously.

*Comment:* These data show the rapid rate with which sodium reaches the sweat. When a correction is made for circulation time, sodium is found to diffuse from the plasma into the interstitial spaces, enter the cells of glomerulus of the sweat glands, be made into sweat, and delivered to the surface of the body in less than sixty seconds. Fig. 17 suggests many interesting concepts of integrations of urinary, sweat, and plasma sodium concentrations. Any

comments based on one experiment would have little significance. More extensive studies will be described at a later date.

#### GENERAL DISCUSSION

The diffusion of sodium through the vascular bed in man behaves in a manner similar to that described by Gellhorn and associates for guinea pigs and dogs.<sup>4-6</sup> For comparison the vascular bed can be divided into at least two areas,  $A_1$  and  $A_2$ , through which diffusion occurs at two very different rates. The more rapid rate of sodium exchange, through  $A_1$ , takes place between the plasma and the intercellular fluid. The slower rate of exchange, through  $A_2$ , occurs between the plasma and such fluids as that of the vitreous body, aqueous humor, cerebrospinal fluid, and probably the intracellular fluid. Inspection of the regression curves shown indicates a third main, and even slower, rate of diffusion which may occur between plasma and bone, dentin, and enamel.<sup>9</sup> This area of the vascular bed through which this occurs might be referred to as  $A_3$ . There is no doubt that there are *many* other differences in rates of diffusion of sodium between the plasma and other structures of the body, the number being limited by the sensitivity of the method of analysis. Under different states of salt intake, nutrition, glandular secretion, vasomotor function, and disease these rates are altered. For example, there is a different rate of diffusion into edema fluid (Fig. 8) from that into ascitic fluid (Fig. 16). A generalized edema state results, therefore, in two areas in the vascular bed with different rates of diffusion. At the present time it is much better to consider the vascular bed as having two main areas with diffusion at a rapid rate and at a slow rate, these being summations of many different rates of diffusion. It is necessary to remember that this may be an oversimplification of the problem and that with further study other rates will be defined.

The rate of the circulation in man is relatively slow when compared with that which would be desirable to insure adequate mixing for proper study of a substance which diffuses as rapidly as sodium. The mixing time in the average person is so long and the diffusion of sodium out of the blood vessels so rapid that in most cases data were obtained which did not lend themselves to detailed mathematical analysis. In some experiments, however, the mixing was rapid and adequate so that a mathematical analysis was possible. Several of these were selected for such a study. The period during the first minute after the injection was not suitable for proper study of diffusion because slow mixing and rapid diffusion complicated the diffusion pattern. The dashed lines in Figs. 2, 8, 9, and 13 show the rapid increase in the concentration of  $\text{Na}^{24}$  in the blood within the first minute or two after the injection of the radiosodium, during which time the regression curves are modified to an unknown extent. The remaining portion of the curves is a more accurate representation of the facts. There is no doubt that if there are any errors, such as those due to slow mixing, they are concerned with factors which tend to make the observed  $b_1$ , the rapid rate of diffusion of sodium, smaller than it actually is. The values for  $b_2$ , the slow rates of diffusion, are most probably near the

true rate since mixing interferes little with the latter portions of the regression curves. Furthermore, since a state of equilibrium had not been fully reached by the end of ninety minutes, the equilibrium values,  $c_{eq}$  of equations (2) and (5), are not the true equilibrium values. Studies<sup>4</sup> with the long-life sodium,  $\text{Na}^{22}$ , in other subjects have shown the true  $c_{eq}$ , the equilibrium constant, to be near those employed in the regression curve formulas, however.

The coefficients,  $a_1$  and  $a_2$ , of the various regression curves are dependent upon such factors as rates of diffusion, size of the areas  $A_1$  and  $A_2$ , and the distribution of the sodium. Therefore,  $a_1$  and  $a_2$  can represent only roughly the degree of distribution of  $\text{Na}^{21}$  in the two compartments of the body, that is, the one which is concerned with a rapid rate of diffusion of sodium and the other with a slower rate. An index of the volume of the blood plasma should be  $c_0$ , the value of  $c_p$  at  $t = 0$ , but because of the difficulties introduced by slow mixing it is difficult to interpret.

The rates of diffusion appear to be quite variable among the individuals of all groups studied (Figs. 1 and 7). The rates of diffusion through areas  $A_1$  and  $A_2$  were definitely greater in the patients with congestive heart failure than in the normal subjects. Because of the marked variations and the relatively small number of subjects studied, it is not possible to be certain of the significance of these differences. However, as indicated previously, this is expected because of the greater absolute quantities of interstitial fluid and, therefore, of sodium in the patients with congestive heart failure. The turnover of the interstitial sodium in the patients with congestive heart failure is essentially half as rapid as in normal subjects, while the plasma sodium turnover is twice as rapid in the patients with congestive heart failure as that in the normal subjects.

The patients with congestive heart failure were too ill to permit the insertion of ureteral catheters into the pelves of the kidneys. A small urethral catheter was placed in the bladder instead. This resulted in a satisfactory study of  $\text{Na}^{24}$  excretion in the urine but did not present the advantage of studying each kidney separately. All sodium clearances for the patients with congestive heart failure represented total clearance, while those in the normal subjects represent unilateral clearance, or essentially one-half of the total clearance (Fig. 6). This should be remembered when examining the illustrations and when comparing the rates of clearance in the normal and abnormal subjects.

The radiosodium was excreted in essentially the same concentration and at the same rate by both kidneys. There was a tendency at times, however, for one kidney to excrete more rapidly than another, but this was varied throughout the period of study. Although the concentration of  $\text{Na}^{24}$  and the rate of sodium clearance tended to vary concordantly, there were sufficient variations to indicate differences in function between two contralateral normal kidneys. Although both are under the same general hormonal or chemical and nervous influences there were individual differences. Most of these differences must result from differences in intrinsic renal function. In some isolated instances these differences were considerable, although the excretion of  $\text{Na}^{24}$  usually differed very little in the two kidneys.

The rate of sodium clearance was much slower in the patients with congestive heart failure than in the normal subjects. This is particularly significant when the rapid transcapillary diffusion rates are considered. It was impossible to evaluate the differences between the two groups of subjects because the patients with congestive heart failure were receiving treatment for the failure. This treatment included dietary restriction of sodium, a procedure normally associated with a decrease in renal excretion of sodium. Nevertheless, these differences are largely due to the congestive heart failure, as evidenced by previous studies<sup>7</sup> with  $\text{Na}^{22}$  and to the fact that three of the patients with a low sodium clearance had not been on sodium restriction for more than twenty-four hours before the study was begun. The long life of  $\text{Na}^{22}$  made it possible to control sodium intake, therapy, diet, and other factors in order to evaluate better the disturbances in sodium excretion in congestive heart failure. Unfortunately, this was not possible in these studies with  $\text{Na}^{24}$ .

Table I shows estimations of sodium space. A sodium space calculated within one or two hours after injection cannot be accurate. This is obvious from the preceding discussion of the rapid rate of diffusion of sodium and the relatively long time required for a state of equilibrium or "complete mixing" of the  $\text{Na}^{24}$  with all of the  $\text{Na}^{23}$  distributed through the body to occur. The inaccuracy of such a calculation is further indicated by Table I. The sodium space calculated from data at the sixtieth minute after injection of the  $\text{Na}^{24}$  was lower than that calculated at the ninetieth minute. Furthermore, radio-sodium cannot be employed to measure extracellular water volume with any degree of accuracy because a fairly large portion of the body sodium is not in the extracellular water. The method becomes even more inaccurate in disease states, especially in those associated with edema. The values of sodium space presented in Table I are intended only to illustrate the possible error of such a calculation and not to present absolute values of sodium space. The lower equilibrium level,  $c_{eq}$ , for the patients with congestive heart failure and the larger values obtained for the estimated sodium space in these patients are quantitative evidence of the large edema fluid volume accompanying congestive heart failure.

The rapid rate and high concentrations with which  $\text{Na}^{24}$  appeared in sweat is further evidence in support of the rapid rate of diffusion of sodium across the vascular wall. The relationships of sodium in the sweat, urine, blood, and edema fluid are under study. The single study presented is not sufficient to warrant any definite physiologic conclusions, although many ideas are suggested.

Finally, it is important to note that the rapid rates of sodium and water diffusion and the tremendous quantities concerned make the problem of the mechanism of edema assume a different pattern. Sufficient evidence has not been gathered yet to make possible a proper appreciation of the role of the rapid and large turnover of sodium and water in edema formation. It is obvious that slight disturbances in sodium diffusion or excretion might result in a rapid and marked accumulation of edema fluid. These factors are now under observation.

## SUMMARY

Normal subjects, patients with congestive heart failure under treatment, and patients with other disease states were studied following a single intravenous injection of  $\text{Na}^{24}$  to learn the rate of diffusion of sodium through the vascular wall and its rate of excretion into the urine and sweat.

Sodium diffuses through the vascular wall with at least two rates, *rapid* and *slow*. These rates of diffusion of sodium in man are similar in quantity and quality to those described by Gellhorn and associates for guinea pigs and dogs. In a normal subject the average rate of diffusion indicated that about 32 per cent of the total plasma sodium diffused out of the blood every minute. This approaches a diffusion across the vascular wall equal to about 18 pounds of sodium or 50 pounds of sodium chloride per day.

The sodium that diffuses at a rapid rate is mainly concerned with an exchange between the plasma and interstitial fluid, while that which diffuses at the slow rate is probably concerned with exchanges between the plasma and such fluids as the vitreous body, aqueous humor, and cerebrospinal fluid. The regression curves observed suggest a possible third or slower rate of sodium diffusion such as might be expected for bone, dentin, and enamel already saturated with sodium.

The individual subjects in all groups showed a considerable spread in the blood and urine concentrations and rates of sodium cleared by the kidneys. The patients with congestive heart failure with treatment, including dietary restriction of sodium, showed a more rapid rate of sodium diffusion from the vascular bed than did the normal subjects. This was also true for a patient with phlebothrombosis receiving only bed rest therapeutically. The rate of turnover of the interstitial fluid sodium was 50 per cent as rapid in the patients with congestive heart failure as in the normal subject, while the plasma sodium of the patients with congestive heart failure turned over twice as rapidly as that of the normal subject.

The rapid rates of diffusion and the large quantities of sodium involved make it necessary to reconsider the role of sodium in edema formation. Relatively slight changes in the rates of sodium diffusion from the blood with a disturbance in its excretion in the urine might result in rapid and marked edema formation.

The rate of sodium clearance in the patients with congestive heart failure was considerably less than that in the normal subjects. These differences were not due entirely to a dietary restriction of sodium intake.

Simultaneous study of each kidney showed a tendency for the renal excretion of sodium to be similar bilaterally. There was sufficient qualitative and quantitative difference, however, to indicate intrinsic renal differences in the excretion of sodium. In some instances these differences were considerable.

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## REFERENCES

1. Hamilton, J. G., and Stone, R. S.: Excretion of Radiosodium in Man Following Intravenous Injection, *Proc. Soc. Exper. Biol. & Med.* 35: 595, 1936-37.
2. Kaltreider, N. L., Meneely, G. R., Allan, J. R., and Bale, W. F.: Determination of Volume of Extracellular Fluid of Body With Radioactive Na (Compared With Thiocyanate), *J. Exper. Med.* 74: 569, 1941.
3. Moore, F. D.: Determination of Total Body Water and Solids With Isotopes, *Science* 104: 157, 1946.
4. Gellhorn, A., Flexner, L. B., and Hellman, L. M.: The Transfer of Sodium Across the Human Placenta, *Am. J. Obst. & Gyneec.* 46: 668, 1943.
5. Gellhorn, A., Merrell, M., and Rankin, R. M.: Rate of Transcapillary Exchange of Sodium in Normal and Shocked Dogs, *Am. J. Physiol.* 142: 407, 1944.
6. Merrell, M., Gellhorn, A., and Flexner, L. B.: Studies on Rates of Exchange of Substances Between Blood and Extravascular Fluid; Exchange of Sodium in the Guinea Pig, *J. Biol. Chem.* 153: 83, 1944.
7. Reaser, P., and Burch, G.: Radiosodium Tracer Studies in Congestive Heart Failure, *Proc. Soc. Exper. Biol. & Med.* 63: 543, 1946.
8. Burch, G. E., and Reaser, P.: Studies in Normal Subjects and Patients With Congestive Heart Failure With the Use of the Long Life Radiosodium, Na<sup>22</sup>. To be published.
9. Hodges, H. C., Koss, W. F., Ginn, J. T., Falkenheim, M., Gavett, E., Fowler, R. G., Thomas, I., Bonner, J. F., and Dessaner, G.: The Nature of the Insoluble Sodium of Bone. The Absorption of Sodium at Forty Degrees by Bone, Dentin, Enamel, and Hydroxyapatite as Shown by the Radioactive Isotope, *J. Biol. Chem.* 148: 32, 1943.
10. Kinsey, V. E., Grant, W. M., Cogan, D. G., Livingood, J. J., and Curtis, B. R.: Sodium, Chloride and Phosphorus Movement and the Eye, *Arch. Ophth.* 27: 1126, 1942.
11. Kinsey, V. E., Grant, W. M., Cogan, D. G.: Water Movement and the Eye, *Arch. Ophth.* 27: 242, 1942.
12. Visser, M., and Carr, C.: The Rate of Entrance of Radiosodium Into the Aqueous Humor and Cerebrospinal Fluid, *Am. J. Physiol.* 142: 27, 1944.

# MANNITOL: KINETICS OF DISTRIBUTION, EXCRETION, AND UTILIZATION IN HUMAN BEINGS

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MANNITOL is in current clinical use as a measure of the rate of glomerular filtration<sup>14</sup> and of the volume of extracellular fluid.<sup>11</sup> It is also recommended as a harmless osmotic diuretic.

The availability of a method<sup>4</sup> for the determination of plasma mannitol, which, especially at low concentrations, is more precise than procedures formerly in use, enabled a study of the distribution, excretion, and utilization of mannitol after intravenous injection in human beings. This study is based on concepts previously developed<sup>5-8</sup> and applied to creatinine, xylose, and galactose. Since these concepts have been presented in detail and verified experimentally, we limit our prefatory discussion to general terms and to specific symbolisms used in this report.

If the rate of urinary excretion,  $y$ , of a substance (milligrams per minute) is proportional to the concentration,  $x$ , of this substance in plasma (milligrams per cubic centimeter), the excretion constant  $A$  can be calculated. This constant  $A$  is usually interpreted as the volume of plasma cleared of the substance per unit time and is called clearance (cubic centimeter per minute). It may be interpreted also as the rate of excretion per unit plasma concentration, or, in short, the specific rate of excretion. When the substance in question is disposed of wholly by renal excretion and when the rate of diffusion of this substance into body fluid is such that the amount lost before equilibration is negligible, the constant  $A$  can be calculated from serial determinations of the concentration of the substance in plasma. The calculation can be made graphically from a plot of plasma concentration  $x$  on semilogarithmic paper. This plot will follow a straight line with slope  $\alpha$ . The initial concentration  $a$  is obtained directly by extrapolating the line to the time of injection. This value  $a$  corresponds to a theoretic concentration of the substance in the plasma under the assumption of mixing and equilibration at the time of injection. The dose,  $G$ , being known, the volume of distribution,  $V$ , of the substance is determined from  $G$  and  $a$ . The excretion constant  $A$  is obtained from  $G$ ,  $a$ , and slope  $\alpha$ . The formulas are collected for convenience in Table I, while Fig. 1 illustrates the procedure.

When the rate of equilibration is such that significant disposal of the substance takes place before equilibrium is established, the values  $V$  and  $A$  must be regarded only as first approximations, identified by a subscript 1, as  $A_1$  and  $V_1$ . If the pre-equilibrium plasma concentrations are known, as in the experiment shown in Fig. 1, the slope  $\beta$  and the extrapolated concentration  $b$  can be determined from the excess of plasma concentration above the line of the slope  $\alpha$ .

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With these new constants a second approximation,  $A_2$ , and a new  $V$ ,  $V_2$ , are then calculated (Table I). The value  $A_2$  measures the total specific rate of disposal of the substance by urinary and other possible pathways and represents total plasma clearance.

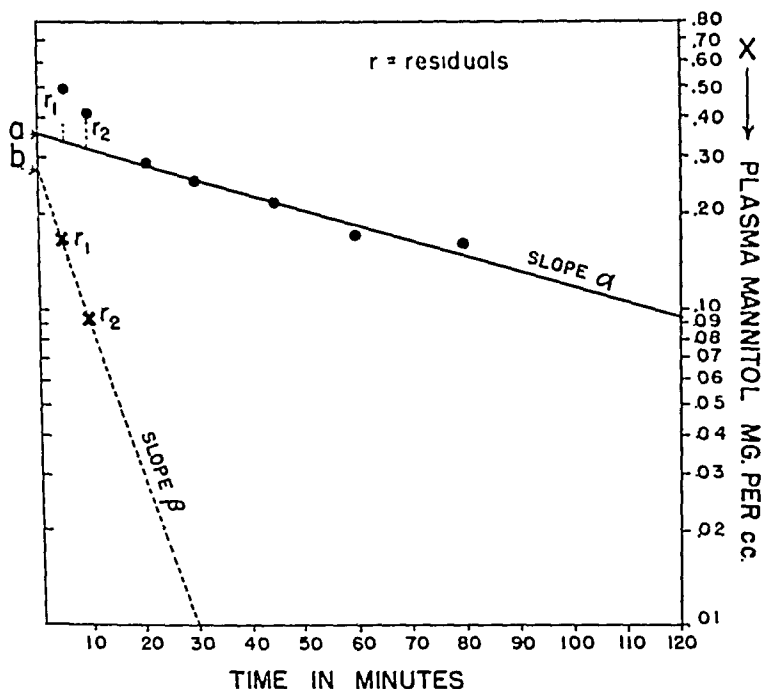


Fig. 1.—Graphic solution of the equation of the plasma disappearance curve,  $X = ae^{-at} + be^{-\beta t}$  (No. 77).

$$a = .355$$

$$a = \frac{\ln 0.355 - \ln 0.10}{113.5} = \frac{1.2669}{113.5} = 0.01116$$

$$b = .285$$

$$\beta = \frac{\ln 0.285 - \ln 0.01}{29.6} = \frac{3.3499}{29.6} = 0.1131$$

The extraordinary pathways of disposal lie either in metabolism or extra-renal excretion. The sum of these processes is referred to herein and in earlier reports as utilization. If the substance (1) is both excreted in the urine and utilized and (2) disappears exponentially from plasma, the former value  $A_1$  must be regarded as the sum of two constants,  $A_s$  and  $B_s$ , of which  $A_s$  is another approximation to the renal plasma clearance and  $B_s$  is the utilization constant. This constant represents the rate of utilization per unit plasma concentration, or, in brief, the specific rate of utilization.

The value of  $A_s$  is calculated from the amount,  $E$ , of the substance which appears in the urine. When the time of urine collection is prolonged and renal function intact, so that excretion is virtually complete in the time of collection,  $A_s$  is calculated as shown in Table I. When the period of collection is short, so that excretion is incomplete, an exponential term is introduced leading to the value  $A_s'$  (Table I).

TABLE I. FORMULAS USED IN THIS REPORT

(1) $y = \Lambda x$	(2) $\int y \, dt = \Lambda \int x \, dt$
(3) $x = ae^{-at} + be^{-\beta t}$	(4) $\Lambda_1 = \frac{\int y \, dt}{\int x \, dt} = \frac{G}{\int ae^{-at} \, dt} = \left( \frac{a}{\alpha} \right)$
(5) $\Lambda_2 = \frac{G}{\int (ae^{-at} + be^{-\beta t}) \, dt} = \frac{G}{\frac{a}{\alpha} + \frac{b}{\beta}}$	
(6) $\Lambda_2 = \frac{E}{\frac{a}{\alpha}}$	(7) $\Lambda_1 = \frac{E}{\frac{a}{\alpha} + \frac{b}{\beta}}$
(8) $\Lambda_1' = \frac{E_t}{\frac{a}{\alpha} (1 - e^{-at})}$	(9) $\Lambda_1' = \frac{E_t}{\frac{a}{\alpha} (1 - e^{-at}) + \frac{b}{\beta} (1 - e^{-\beta t})}$
(10) $\frac{\Lambda_2}{\Lambda_1} = \frac{\Lambda_1}{\Lambda_2} = \frac{E}{G}$	(11) $B_1 = \Lambda_2 - \Lambda_1$
(12) $V_1 = \frac{\Lambda_1}{\alpha}$	(13) $V_2 = \frac{\Lambda_2 (\beta^2 a + a^2 b)}{(\beta a + ab)^2}$
(14) $\frac{\Lambda_1}{\Lambda_2} = 1 + \frac{\alpha}{\beta} \cdot \frac{b}{a}$	(15) $\frac{V_1}{V_2} = 1 + 2 \frac{\alpha}{\beta} \cdot \frac{b}{a}$ approximately

The formulas used herein and in earlier reports are summarized in this table for convenience. The meanings of the symbols are explained in the text.

G, Quantity injected in milligrams.

E, Total urinary excretion, milligrams.

E<sub>t</sub>, Quantity excreted in urine in time, t.

In the calculations of  $\Lambda_1$  and  $\Lambda_1'$ , the diffusion of the substance is neglected. If both diffusion and extraordinary disposal are taken into consideration, a value,  $\Lambda_4$  (and also  $\Lambda_4'$  for a short period of collection), can be calculated as shown in Table I. The value  $\Lambda_4$  is equal to renal plasma clearance of a substance which is excreted in urine and which is also utilized and subject to pre-equilibration disposal.

Successive calculations of these different values of  $\Lambda$  give explicitly the errors introduced in the estimate of renal clearance. The ratio  $\frac{\Lambda_1}{\Lambda_2}$  gives the relative error introduced in the estimate  $\Lambda_1$  by neglecting diffusion. The ratios  $\frac{\Lambda_1}{\Lambda_2} = \frac{\Lambda_2}{\Lambda_4}$  give the relative error introduced in the estimates  $\Lambda_1$  and  $\Lambda_2$  by assuming no utilization. The ratio  $\frac{\Lambda_1}{\Lambda_4}$  gives the relative error introduced in the estimate  $\Lambda_1$  by neglecting diffusion and by assuming no utilization.

The utilization constant  $B$  already defined is obtained from the difference  $(\Lambda_2 - \Lambda_4)$  (Table I).

#### PROCEDURE

Seventy experiments were performed on fifty-six subjects, thirty-nine of whom suffered from some form of renal disease. Twenty cubic centimeters of a

25.5 per cent solution of mannitol\* were rapidly injected intravenously. Plasma samples were obtained after equilibration in some experiments and both before and after equilibration in other experiments. The number of plasma samples varied from three to seven. Urine was collected for short periods (averaging 105 minutes) and for long periods (nine to twelve hours). The distribution of procedures is shown in Table II.

TABLE II

PLASMA SAMPLING	URINE COLLECTION			TOTALS
	NONE	SHORT	LONG	
After equilibration	36	13	7	56
Before and after equilibration	2	7	5	14
Total	38	20	12	70

Data from one of the five experiments with long urine collections in which plasma was obtained before and after equilibrations are shown in Fig. 1, from which the course of other experiments can be visualized. In each of ten subjects the experiments were repeated once, and in each of two other subjects the experiments were done three times. Two of the repeated experiments cannot be averaged because of changes in the subject's renal excretory function.

Plasma mannitol clearance,  $C$ , was determined in patients independently of this procedure on thirty occasions (Table IV). These determinations were done by the conventional method of intravenous infusion, plasma sampling, and urine collection by catheterization and by bladder washing. A value termed  $C'$  was calculated from the curve of plasma concentrations and concurrent excretion of mannitol in voided urine in five experiments. This value is equivalent to conventional clearance determination, but, in contrast with  $C$  in this series, the determination  $C'$  is simultaneous with the experiment rather than an independent observation.

## RESULTS

*Volume of Distribution.*—Mean  $V_i$  in the whole group is 14.2 liters, while mean body weight,  $W$ , is 66.7 kilograms, mean surface area,  $S$ , is 1.74 square meters, and mean developmental level,  $D.L.$ ,<sup>16</sup> is 166. The correlation coefficient between  $V_i$  and  $W$  is 0.49; that between  $V_i$  and  $S$ , 0.53; and between  $V_i$  and  $D.L.$ , 0.53. The relation between  $V_i$  and  $D.L.$  is shown in Fig. 2, which demonstrates that, on an average,  $V_i$  is greater in patients than in normal subjects at equal developmental levels. The differences in the means of  $V_i$  between patients (14.9 liters) and normal subjects (12.6 liters) is 2.3 liters, which is found to be 2.8 times the standard error of the difference and is, therefore, significant. The importance of the difference is still more apparent when it is related to body weight or developmental level in the two groups, mean  $W$  being 65.9 kilograms in patients and 68.8 kilograms in the normal group and mean  $D.L.$  being, respectively, 164 and 170.

\*We acknowledge the generosity and interest of Dr. Carl Beyer and Dr. William Boger, of Sharp & Dohme, Inc., Philadelphia, Pa., and the skillful assistance of Dr. L. Underwood and Miss E. Davy, R.N.

The second approximation of volume of distribution,  $V_2$ , is calculable in those cases in which plasma mannitol was determined before equilibration. In this group  $V_1$  averages 13.8 liters and  $V_2$ , 12.3 liters (Table III). The difference is 1.5 liters and the ratio  $V_1:V_2$  is 1.12. The excess of the ratio  $V_1:V_2$  over unity measures the error in the estimate of volume of distribution occasioned by

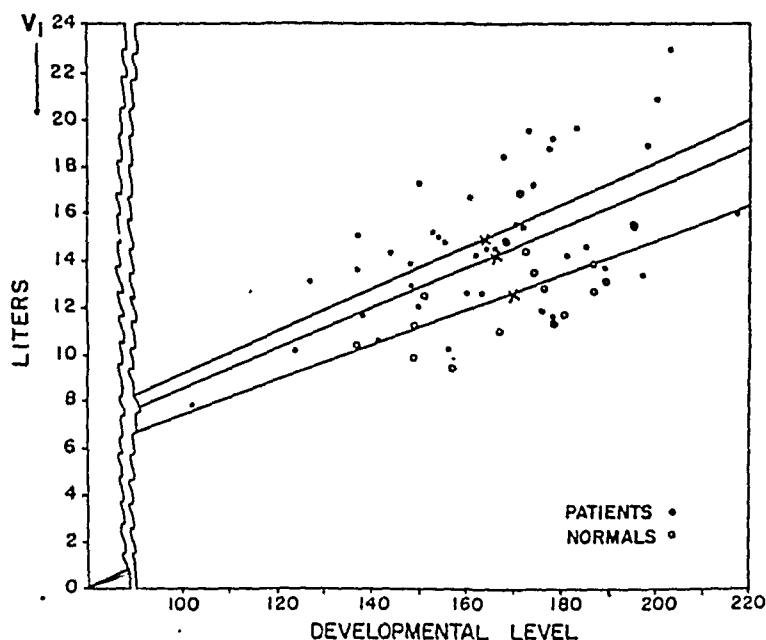


Fig. 2.—Relation between  $V_1$  and developmental level,  $D.L.$ , in patients and in normal subjects. The means for each group are indicated as  $X$ . These points are joined to zero by straight lines. The upper line indicates the relation of  $V_1:D.L.$  in patients; the lower line, the relation in normal subjects; and the middle line, that in the whole group.

TABLE III. CONSTANTS OF THE EQUATION OF PLASMA MANNITOL DISAPPEARANCE

$$x = ae^{-\alpha t} + be^{-\beta t} \quad \text{AND DERIVED VALUES OF } P, V_1 \text{ AND } V_2$$

NO.	a	$\alpha$	b	$\beta$	P	$V_2$	$V_1$
60	35.5	0.0049	40.0	0.290	6.76	13.8	14.4
61	36.8	0.0043	15.0	0.082	9.85	13.3	13.9
62	30.4	0.0056	25.0	0.153	9.21	15.8	16.8
63	43.1	0.0037	40.0	0.150	6.14	11.3	11.8
64	49.5	0.0075	35.0	0.178	6.03	9.7	10.3
65	37.6	0.0092	34.5	0.096	7.1	11.7	13.6
66	36.0	0.0095	19.2	0.100	9.2	12.9	14.2
68	35.2	0.0092	59.0	0.076	5.4	10.2	14.5
69	22.3	0.0097	14.0	0.091	14.0	20.3	22.9
77	35.5	0.0112	28.5	0.113	8.0	12.4	14.4
78	40.5	0.0117	44.5	0.119	6.0	10.4	12.6
79	38.2	0.0121	23.0	0.098	8.3	11.7	13.4
80	52.0	0.0131	36.5	0.128	5.8	8.6	9.8
81	45.6	0.0112	42.5	0.155	5.8	10.5	11.2
Means					7.68	12.33	13.8

Constants obtained in experiments in which plasma mannitol concentrations were known before and after equilibration. The value  $P$  (Dominguez) is the volume into which injected mannitol diffuses so rapidly that it is identified in earlier reports as equivalent plasma volume. In these experiments the volume  $P$  averages about 2.7 times true plasma volume as estimated from body weight and 0.62 times the final volume of distribution,  $V_2$ . The significance of the other constants is explained in the text.

neglecting diffusion prior to equilibration (Table I).  $V_2$  is, therefore, the more exact measure of volume of distribution. If this value of  $\frac{V_1}{V_2}$  holds for the whole group,  $V_2$  averages 13.3 liters (20 per cent of body weight) in patients and 11.3 liters (16 per cent of the body weight) in normal subjects.

*Equilibration of Mannitol in Body Fluid.*—Since mannitol is subject to continuous disposal, the equilibration of mannitol in body fluids is dynamic. After a single injection, the first gradient is from plasma to interstitial fluid, and the second, from body fluid to plasma, from which mannitol is excreted in urine. The time of equilibration marks completion of the first phase, after which disappearance of mannitol from plasma becomes simply exponential. Plasma samples were obtained at or about twenty minutes after injection in thirty-nine subjects. The weighed mean time of equilibration is twenty-seven minutes, equilibrium being established at twenty minutes in nineteen experiments, at twenty-five in one, at thirty in ten, and at forty in nine. In four experiments in which the first blood sample was taken at thirty minutes, equilibration had already been established. The mean time of equilibration in the fifteen experiments in which blood samples were obtained shortly after injection (as in Fig. 1) was twenty-eight minutes, the times being twenty minutes in six, twenty-five minutes in one, thirty minutes in three, thirty-five minutes in four, and forty-five minutes in one.

Thus, mannitol was equilibrated in body fluids at a mean interval of twenty-seven to twenty-eight minutes after injection, but it is well to bear in mind that in ten of fifty-eight experiments the time of equilibration was forty minutes or more.

*Renal Excretion.*—The first approximation of the specific rate of mannitol disposal  $A_1$  is compared with the independently determined renal plasma clearance,  $C$ , in Table IV. The ratio of the mean of  $A_1$  and  $C$  in this group is 1.44. This estimate of the ratio  $A_1:C$  may be somewhat affected by changes in function between measurements of  $A_1$  and  $C$ . But the inequality of the means indicates that a considerable portion of injected mannitol is disposed of by avenues not taken into account in assuming the equivalence of  $A_1$  and  $C$  in the estimation of  $A_1$ . There must be loss before equilibration, or utilization before and after equilibration, or a combination of these factors.

That disposal before equilibration should not be as great as the excess over unity of  $A_1:C$  indicates is suggested by the rapidity of mannitol diffusion. The effect of diffusion from plasma on the estimate of volume of distribution is noted in the foregoing in comparing  $V_1$  and  $V_2$ . Similarly, the effect of this diffusion on the estimate of specific disposal rate is observed in the ratio of mean  $A_1:A_2$  (Table V) which is 1.08, an excess over unity of the same order as that of the ratio  $V_1:V_2$ . Loss of injected mannitol before equilibration is, therefore, not negligible.

However, if such loss were the only factor determining the excess of  $A_1$  over  $C$ , the ratio  $A_2$  to  $C'$  would be unity. Actually, the ratio of the means is 1.23 (Table V), from which it follows that the specific rate of disposal of injected mannitol involves also a considerable utilization of this substance.

TABLE IV. COMPARISON OF THE MANNITOL CLEARANCE  $C$  TO THE FIRST APPROXIMATION  $A_1$  TO THIS CLEARANCE

C SMALLER THAN 70			C GREATER THAN 70		
NO.	$A_1$	$C$	NO.	$A_1$	$C$
2	95	30	3	182	121
4	92	67	5	157	81
7	56	40	14	145	80
8	64	68	22	109	88
9	60	60	25	132	85
11	67	48	28	103	78
19	58	35	35	153	140
20	82	54	44	139	88
27	103	60	46	163	125
29	81	64	51	188	124
33	72	30	65	125	83
34	55	23	66	135	97
38	23	21			
43	27	17			
60	71	66			
62	94	50			
63	44	28			
64	77	50			
Mean	67.9	46.2		144.3	99.7

Mean  $A_1 = 1.14 \times$  mean  $C$  in patients whose level of  $C$  is less than 70, and 1.15 when  $C$  is greater than 70. The mean of the ratios  $A_1:C$  for patients is 1.62 when  $C$  is less than 70 ( $S.D.$ , 0.55) and 1.17 ( $S.D.$ , 0.23) when  $C$  is greater than 70. The difference between these means is not significant (difference/standard error of difference is less than 1). The greater variability of data in patients in whom  $C$  is less than 70 is attributed in part to changes in function which may have occurred in the interval between measurements of  $A_1$  and  $C$ . Such changes are less marked when function is initially close to normal with  $C$  greater than 70.

TABLE V. SUCCESSIVE APPROXIMATIONS TO THE CLEARANCE OF MANNITOL COMPUTED FROM THE PLASMA CURVE ALONE ( $A_1$  AND  $A_2$ ), FROM THE PLASMA CURVE AND SHORT INTERVAL OF URINE COLLECTION ( $A_2'$  AND  $A_3'$ ) AND FROM THE PLASMA CURVE AND LONG INTERVAL OF URINE COLLECTION ( $A_3$  AND  $A_4$ ). COMPARISON WITH INDEPENDENT CLEARANCE  $C$  AND SIMULTANEOUS CLEARANCE  $C'$ 

NO.	$A_1$	$A_2$	$A_2'$	$A_3$	$A_3'$	$A_4$	$C'$	$C$
60	71	69	66	-	62	-	-	66
62	94	91	50	-	47	-	-	50
63	44	43	44	-	10	-	-	28
64	77	75	48	-	15	-	-	50
65	125	115	56	-	48	-	-	83
66	135	128	78	-	71	-	-	97
77	161	149		137		126	125	
78	147	133		107		96	100	
79	162	150		137		128	111	
80	128	120		111		104	99	
81	125	117		116		108	112	
Mean	145	134		122		112	109	

Data from patients (Nos 60 to 66) and normal subjects (Nos 77 to 81). Data from patients are not averaged because of their variability. The means of the ratios of  $A$  in normal subjects are as follows:  $A_1:A_2$ , 1.29;  $A_2:A_3$ , 1.20;  $A_3:A_4$ , 1.08;  $A_1:C'$ , 1.03;  $A_1:A_2$ , 1.08;  $A_1:A_3$ , 1.19;  $A_1:C$ , 1.33. The specific rate of utilization  $B_1'$  in experiments with short periods of urine collection averages 34 and is widely variable. In normal subjects, with prolonged urine collection, mean  $B_1 = 21$ .

When this utilization is taken into account, as it is in calculating  $A_3$  and  $A_3'$ , neglecting pre-equilibrium disposal, the means of these values only slightly exceed the means of  $C$  and  $C'$  (Table V) (ratio of mean  $A_3:C'$  is 1.12). This excess over unity measures the degree to which the estimate of renal excretion of mannitol ( $A_3$  or  $A_3'$ ) is affected by pre-equilibration disposal. When this loss is also accounted for, as it is in calculating  $A_4$  and  $A_4'$ , the correspondence

of  $A_1$  and  $A_1'$  with  $C$  and  $C'$  is seen to be close (Table V) (ratio of mean  $A_1:C'$  is 1.03). The discordance of three ratios  $A_1:C$  (Nos. 63, 65, and 66) can be attributed to changes in renal function which may have occurred in the interval between the determination of  $C$  and that of  $A_1$ . This supposition is supported by the fact that the concordance with the simultaneous observations  $C'$  is nearly exact.

Thus, specific rate of renal mannitol excretion obtained from serial determinations of plasma mannitol as  $A_1$  or  $A_1'$  corresponds with values for renal plasma mannitol clearance, measured as  $C$  or  $C'$ . Both utilization and diffusion must be taken into account in the estimation of renal mannitol clearance from serial analyses of plasma after injection of mannitol.

*Utilization.*—The difference ( $A_1 - A_1'$ ) establishes the existence of mannitol utilization and measures the specific rate of this utilization,  $B$ . Direct evidence of this extraordinary disposal is obtained (Table VI) from observation of urinary excretion of mannitol over a long interval of time in subjects with normal or nearly normal renal functions. The mean recovery of injected mannitol is 79 per cent. Recoveries of 81 and 89 per cent were obtained by Smith, Finklestein, and Smith<sup>14</sup> in ten and one-half hours of urine collection, and recoveries of 80, 85, and 90 per cent in twenty-four hours by Berger, Farber, and Earle.<sup>2</sup>

TABLE VI. RECOVERY OF MANNITOL IN THE URINE; 5,100 MG. INJECTED

SUBJECT	TIME (MIN.)	AMOUNT OF MANNITOL (MG.)	PER CENT RECOVERED
70	720	3,595	70.5
71	720	4,329	84.9
72	660	4,108	80.5
73	660	3,172	62.2
74	660	3,843	75.4
75	720	3,852	75.5
76	720	3,986	78.2
77	540	4,331	84.9
78	720	3,690	72.4
79	785	4,350	85.3
80	540	4,411	86.5
81	720	4,702	92.2
Mean	680.4		79.04

The validity of a nine- to twelve-hour urine collection as a measure of total urinary excretion can be shown by the following example (No. 77). The value of  $a$  is found as 0.355 mg. per cubic centimeter;  $\alpha$ , .0112;  $b$ , .285;  $\beta$ , .113;  $t$ , 540 minutes;  $\alpha t$ , 6.048;  $\beta t$ , 61.02; and  $1 - e^{-6.048}$ , .99764. Amount excreted in 540 minutes is 4,331 milligrams. Assuming the 540 minute excretion to be total excretion,  $E$ , 4,331 =  $A_1$  (34.22) and  $A_1 = 126.6$  cubic centimeters per minute. Taking now 4,331 only as the amount excreted,  $E_t$ , in 540 minutes, and calculating  $A_1'$ , as is done when urinary excretion is incomplete, we have  $4,331 = A_1'$  ( $31.70 \times .99764 + 2.52$ ) from which  $A_1' = \frac{4331}{34.15} = 126.8$  cubic centimeters per minute. The correspondence of  $A_1$  and  $A_1'$  shows that excretion in 540 minutes is a close measure of total excretion. Actually, with  $A_1' = 126.8$ , the total cal-

culated excretion will be  $126.8 \times 34.22 = 4,339$  milligrams. The difference between this and the amount excreted in 540 minutes is only 8 mg., or 0.2 per cent of  $E$ .

Indeed, when  $\alpha t$  is greater than 4.667, the factors  $1 - e^{-\alpha t}$  and  $1 - e^{-\beta t}$  can be neglected, being less than 1 per cent of unity. As a matter of fact, the smallest value of  $\alpha t$  in the experiments of Table VI is 5.83. Consequently, the amounts excreted in the experiments of Table VI are less than 1 per cent smaller than the total excretion, and the mean recovery of 79 per cent can be considered equal to total recovery of injected mannitol.

The specific rate of mannitol utilization is measured as  $B$ . In experiments of short duration, this constant is estimated as 34, mean of  $B_1'$ , and in experiments with long urine collection, as 21, mean of  $B_2$  (Table V). The greater variability of data in the shorter experiments leads us to believe that the estimate of  $B$  from the longer experiments ( $B_2$ ) is the more representative of the specific rate of mannitol utilization in human beings.\*

#### DISCUSSION

The present investigation was aimed at evaluating renal function by the disappearance of intravenously injected mannitol from plasma without collection of urine in the manner suggested by Barnett<sup>1</sup> for inulin and by Newman, Bordley, and Winternitz<sup>11</sup> for mannitol.

In proposing this procedure as a measure of glomerular filtration, the latter authors note that it is based on the assumptions, "(1) that mannitol is dissolved in a space approximating the extracellular fluid of the body; (2) that an equilibrium of concentration is established throughout the fluid; and (3) that loss of mannitol is only through excretion by the kidneys." The first of these assumptions is supported by our data, since the volume  $V_2$  corresponds well with other estimates of extracellular fluid volume. The third assumption is negated by the demonstrated utilization of mannitol. The view implicit in the second assumption that excretion of mannitol before equilibration is negligible is also controverted, since neglect of this disposal introduces errors of about 10 per cent in the estimates of  $V$  and  $\Delta$ . It is, therefore, impossible to arrive at an accurate estimate of renal plasma mannitol clearance when only the dose injected,  $G$ , and the slope,  $\alpha$ , are known. It has recently been shown<sup>2, 4, 10, 13</sup> that the plasma clearance of mannitol is about 90 per cent of that of substances presumably excreted by glomerular filtration without tubular reabsorption.

However, with the additional safeguards<sup>6</sup> of a knowledge of pre-equilibrium plasma concentrations and of the amount excreted, renal plasma mannitol clearance can be calculated as  $\Delta_2$  or  $\Delta_1'$ . Unfortunately for convenience the procedure then requires at least four and preferably five plasma samples, and, because of the individual variability of the specific utilization constant  $B$ , a prolonged collection of urine.

\*The ratio of the clearance determined by the infusion method of Earle and Berliner<sup>9</sup> to conventional plasma mannitol clearance is found by Berger, Farber, and Earle<sup>2</sup> to be 1.12. If renal plasma mannitol clearance of these subjects is taken as 110, the specific rate of utilization is  $(110 \times 1.12 - 110) \approx 13$ .



The behavior of intravenously injected mannitol in human beings resembles that of xylose in dogs.<sup>9</sup> Like xylose, mannitol is distributed in a comparable volume of body fluid, is largely excreted in the urine by glomerular filtration and slight reabsorption,<sup>12</sup> and is also subject to utilization. On the basis of body weight, the utilization constant  $B$  of mannitol in normal human beings is 0.30, while that of xylose in dogs is 0.97. This difference in the constants emphasizes the value of the calculation of  $B$  in estimating utilization. The mean per cent recovery of xylose in dogs averages 60 per cent and that of mannitol in human beings, 80 per cent, from which it appears that the two substances are utilized in comparable proportion. However, the specific rate of utilization of xylose per unit body weight is three times that of mannitol.

It is interesting to note that in dogs the specific rate of utilization of sorbitol, the isomer of mannitol, is even greater than that of xylose. If we assume a body weight of 15 kilograms and an  $A$  of 35 in the experiments of Todd, Myers, and West,<sup>13</sup> the estimate<sup>5</sup> of  $B$  for sorbitol is 3.5 per kilogram of body weight.

The pathway of utilization of mannitol is unknown. It seems unlikely that mannitol should be subject to significant extrarenal excretion; therefore, utilization of mannitol, like that of xylose, may be taken as due to metabolic conversion.

Most published studies on the metabolism of mannitol are based on feeding experiments. These studies have shown that, in the rat, ingestion of mannitol leads to storage of liver glycogen.<sup>3, 15</sup> In the rabbit, mannitol does not relieve the symptoms of insulin shock.<sup>3</sup> Since mannitol is readily subject to bacterial degradation, it has been assumed that most of the metabolic utilization of ingested mannitol is due to absorption of products of bacterial action. However, the comparatively rapid utilization of mannitol we describe suggests that the results of feeding experiments reflect parenteral conversion rather than enteral breakdown.

#### SUMMARY AND CONCLUSIONS

The disposal of a single intravenous injection of mannitol in human beings is described on the basis of concepts formerly applied to creatinine, xylose, and galactose. The method of calculation is presented.

The volume of distribution of mannitol in body fluids is estimated as 16 per cent of body weight in normal subjects and 20 per cent in patients suffering from cardiac and renal disease. When no allowance is made for disposal of mannitol before equilibration in body fluids, the estimates of distribution volume are increased by 12 per cent. The time of equilibration of mannitol in body fluids averages 27.5 minutes, but in ten of fifty-eight experiments this interval was prolonged to forty minutes or more.

Utilization of mannitol, presumably metabolic, is shown to proceed at a rate which, like the specific rate of excretion, can be expressed as a specific rate of utilization in milligrams per minute per unit plasma concentration. This specific rate is estimated as about 21 in normal human beings. In subjects with normal renal function, excretion of injected mannitol is completed

in nine to twelve hours. The proportion excreted in this time averages 79 per cent of the amount injected.

The estimate of renal plasma mannitol clearance from the exponential plasma disappearance curve and the dose alone after a single intravenous injection is inadequate. In normal subjects the error is found to be about 30 per cent when no allowance is made for pre-equilibration disposal and for utilization. The error is reduced to 20 per cent by taking into account pre-equilibrium disposal, and to about 10 per cent when allowance is made for utilization. Although variability of data is considerable in patients suffering from diseases which alter renal function, the mean error of the estimate of mannitol clearance from the plasma disappearance curve is negligible when allowances are made for both utilization and pre-equilibration disposal. In practice, this estimate requires analysis of serially obtained plasma samples and of urine collected over a long interval.

#### REFERENCES

1. Barnett, H. L.: Renal Physiology in Infants and Children; Method for Estimation of Glomerular Filtration Rate, *Proc. Soc. Exper. Biol. & Med.* 44: 654, 1940.
2. Berger, E. Y., Farber, L. J., and Earle, D. P., Jr.: Renal Excretion of Mannitol, *Proc. Soc. Exper. Biol. & Med.* (In Press.)
3. Carr, C. J., and Krantz, J. C.: Sugar Alcohols: Fate of Polygalitol and Mannitol in Animal Body, *J. Biol. Chem.* 124: 221, 1938.
4. Corcoran, A. C., and Page, I. H.: Determination of Mannitol in Plasma and Urine, *J. Biol. Chem.* (In Press.)
5. Dominguez, R.: Studies of Renal Excretion of Creatinine; Volume of Distribution, *Proc. Soc. Exper. Biol. & Med.* 31: 1146, 1934.
6. Dominguez, R., Goldblatt, H., and Pomerene, E.: Kinetics of Excretion and Utilization of Xylose, *Am. J. Physiol.* 119: 429, 1937.
7. Dominguez, R., and Pomerene, E.: On Renal Excretion of Xylose, *J. Clin. Investigation* 13: 753, 1934.
8. Dominguez, R., and Pomerene, E.: Kinetics of Disappearance of Galactose From Plasma After Rapid Intravenous Injection, *Am. J. Physiol.* 141: 368, 1944.
9. Earle, D. P., Jr., and Berliner, R. W.: Simplified Clinical Procedure for Measurement of Glomerular Filtration Rate and Renal Plasma Flow, *Proc. Soc. Exper. Biol. & Med.* 62: 262, 1946.
10. Hoobler, S.: Personal communication, 1947.
11. Newman, E. V., Bordley, J., III, and Winternitz, J.: Interrelationships of Glomerular Filtration Rate (Mannitol Clearance), Extracellular Fluid Volume, Surface Area of Body, and Plasma Concentration of Mannitol; Definition of Extracellular Fluid Clearance Determined by Following Plasma Concentration After Single Injection of Mannitol, *Bull. Johns Hopkins Hosp.* 75: 253, 1944.
12. Shannon, J. A.: Tubular Reabsorption of Glucose in Normal Dog, *Am. J. Physiol.* 122: 775, 1938.
13. Smith, H. W.: Personal communication, 1947.
14. Smith, W. W., Finkelstein, N., and Smith, H. W.: Renal Excretion of Hexitols (Sorbitol, Mannitol, and Dulcitol) and Their Derivatives (Sorbitan, Isomannide, and Sorbide) and of Exogenous Creatinine-like Chromogen in Dogs and Man, *J. Biol. Chem.* 135: 231, 1940.
15. Todd, W. R., Myers, J., and West, E. S.: On Metabolism of Sorbitol and Mannitol, *J. Biol. Chem.* 127: 275, 1939.
16. Wetzol, N. C.: Growth, Medical Physics (O. Glasser, editor), Chicago, 1944, The Year Book Publishers, Inc., p. 513.

## STUDIES IN SERUM PROTEINS

### I. THE CHEMICAL ESTIMATION OF ALBUMIN AND OF THE GLOBULIN FRACTIONS IN SERUM

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THE increasing clinical significance being attached to the various components of the serum proteins as revealed by electrophoresis necessitates a simple, rapid procedure for estimating these fractions. The characteristics of the Tiselius electrophoresis apparatus make it impractical for most clinical laboratories. As a result, fractionation studies cannot be performed in many cases where they would be desirable.

A simple chemical fractionation method which approximated the results obtained by electrophoresis is reported. This chemical procedure requires no special equipment and can be performed in several hours of actual working time using serum samples of nominal size.

#### MATERIALS AND METHODS

Four human sera, one normal and three abnormal, were fractionated by both electrophoretic and chemical methods. Five samples of animal plasma were also studied; these were obtained as desiccated powders and were reconstituted with distilled water. In the case of the animal plasma samples it was necessary to undertake a preliminary removal of fibrinogen; this was done by the addition of purified clotting thrombin. Three of the animal samples were of sheep plasma, and one each of equine and porcine plasma.

Analyses‡ on the human sera and on the animal plasma samples were carried out electrophoretically; in calculating the percentage composition of the fractions of these samples (Table II) a suitable correction has been made for fibrinogen.

Chemical procedures utilized for determining the various protein fractions were as follows:

1. Total protein was determined by the biuret reaction as modified by Weichselbaum.<sup>1</sup>

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‡Analyses on human sera carried out by Dr. Donald Buchanan; those on animal plasma samples, by Dr. J. D. Perrings.

We are greatly indebted to Dr. J. D. Perrings and Dr. Donald Buchanan, of the Department of Medicine, University of Chicago, for their kind cooperation in making it possible to check our human sera analyses runs, and to Dr. R. L. Kutz and Dr. J. D. Perrings of the Biochemical Laboratories, Armour and Company, who provided us with liberal samples of desiccated animal plasma of precisely known electrophoretic composition.

2. Albumin plus alpha globulin is equivalent to the "albumin" fraction obtained by employing Kingsley's modification of Howe's method to precipitate "globulin."<sup>2</sup> The estimation was finally performed by the biuret method.
3. Albumin values were obtained by the methanol fractionation procedure of Pillemer and Hutchinson.<sup>3</sup> The methanol filtrate was analyzed for protein by the biuret reaction.
4. Gamma globulin was separated from serum by a modification of the method of Cohn and co-workers.<sup>4</sup> A solution of the precipitating reagent was prepared by adding 2.5 ml. of 1 N. sulfuric acid to 100 ml. of 2.78 M. ammonium sulfate. Five-tenths milliliter of serum was placed in a graduated 10 ml. Kolmer centrifuge tube. Four milliliters of precipitating solution were then placed in a doubly tied cellophane bag suspended in the tube and centrifuged until 0.5 ml. of ammonium sulfate solution had been added to the serum. The rate of centrifugation was adjusted so that about two hours were required. The cellophane bag was then removed. After standing overnight, the tube was again centrifuged until the precipitate was well packed. The supernatant was discarded and the precipitate taken up in 0.9 per cent NaCl solution. The protein concentration, consisting almost entirely of gamma globulin,<sup>4</sup> was estimated by the turbidometric method of Ayer, Dailey, and Fremont-Smith.<sup>5</sup>

Care should be taken to approximate the end point as closely as possible. However, the end point should be passed slightly, an amount of serum exactly equal to the excess of ammonium sulfate added should be pipetted into the centrifuge tube. The serum added is then mixed well with the supernatant and the tube warmed to 35 to 40° C. for thirty minutes, following which it is permitted to stand overnight and treated in the usual fashion.

5. The value for beta plus gamma globulin was obtained by subtracting the value for albumin plus alpha globulin from total protein values (Step 1 minus Step 2).
6. Beta globulin was estimated by subtracting the value obtained for gamma globulin (Step 4) from the value obtained for beta plus gamma globulin (Step 5).
7. Alpha globulin concentration was obtained by subtracting the value for albumin (Step 3) from that of albumin plus alpha globulin (Step 2).

These determinations can be accomplished in three hours of actual work. However, since the gamma globulin precipitation requires standing overnight twelve to eighteen hours are needed from the time the samples are received. It is preferable that the determinations be carried out in duplicate. For the complete analyses in duplicate 6.5 ml. of serum are required.

#### RESULTS AND DISCUSSION

A consideration of the method outlined will show that its operation fundamentally depends upon the assumption that the "albumin" found by the Howe method of sodium sulfate precipitation actually is equivalent to the electro-

phoretically determined true albumin plus alpha globulin, and that the "globulin" of the Howe method actually is equivalent to beta plus gamma globulin. This assumption was originally based upon a review of the older papers in which sodium sulfate and electrophoretic fractionation were compared.<sup>6, 7</sup> While the present study was in progress, Petermann, Young, Hoagness, and Rhoads<sup>8</sup> also observed the coincidence in values between the Howe "albumin," on one hand, and the sum of the electrophoretic values for albumin and alpha globulin on the other.

The data obtained in the course of this study appear to justify this assumption. Table I lists the values obtained for various serum components by the chemical and the electrophoretic method in human subjects. In both Table I and Table II good correspondence is shown between the Howe "albumin" (albumin plus alpha globulin obtained by the chemical method) and the electrophoretic values for albumin plus alpha globulin.

No difficulty was encountered in obtaining duplicate checks of the values for total protein, albumin plus alpha globulin, beta plus gamma globulin, and gamma globulin. However, we agree with Petermann and co-workers<sup>8</sup> in emphasizing that to obtain reliable and reproducible results for albumin, the most meticulous care must be used in the preparation of the reagents, in avoiding evaporation, and in maintaining all components of the system at 0° C. throughout the precipitation and filtration process. Because of the delicacy of the Pillemer and Hutchinson method,<sup>3</sup> consideration is being given to the possibility of substituting the albumin method suggested by Chow<sup>9</sup> which he has shown also to give values for albumin which approximate electrophoretic values.

In Table II we have compared all average values obtained by the chemical method with those obtained by electrophoresis. It can be seen that the average difference between the two methods does not exceed 10 per cent for any fraction. Electrophoretic values depend on the particular buffer used and some fractions may vary as much as 60 per cent in successive determinations on the same sample with different buffers. The agreement between the electrophoretic and precipitation methods appears to be satisfactory.

The agreement between individual electrophoretic and chemical results<sup>\*</sup> was somewhat poorer for the animal sera than for the human. It appears likely that this resulted from the necessity of removing fibrinogen by the use of clotting thrombin before the samples could be analyzed.<sup>10</sup>

In addition to its general availability, the chemical method has one advantage not shared by the Tiselius procedure. Since the biuret method employed for the individual determinations estimated protein alone, other substances of lipid or carbohydrate nature which migrate with protein fractions are not measured. In the Tiselius apparatus, values for such protein-bound substances are included in calculating the percentage composition of the various fractions. On the other hand, the Tiselius method provides a direct check on the purity of the individual fractions in the schlieren diagram; and it also permits determination of subfractions of the major globulin fractions.

<sup>\*</sup>We have been requested to withhold individual electrophoretic analyses of the animal samples as these form a portion of studies to be published separately.

TABLE I. COMPARISON OF PROTEIN FRACTIONATION VALUES OBTAINED BY ELECTROPHORESIS AND CHEMICAL PRECIPITATION IN HUMAN SERA

SERUM	DIAGNOSIS	FRACTIONATION METHOD	TOTAL PROTEIN (GM. %)	ALBUMIN (GM. %)	GLOBULIN (GM. %)		
					ALPHA	BETA	GAMMA
1	Normal	Chemical	7.2	1.1	1.4	0.11	1.4
		Electrophoresis	7.3	3.9	1.3	0.77	1.3
2	Hypogammaglobulinemia with lymphopenia and lymphatic atrophy and bronchiectasis	Chemical	3.5	2.0	0.9	0.17	0.13
		Electrophoresis	3.6	2.1	0.9	0.11	0.15
3	Chronic glomerulonephritis, nephrotic phase	Chemical	4.0	0.8	1.2	1.7	0.33
		Electrophoresis	1.0	0.8	1.6	1.1	0.25
4	Sarcoidosis?	Chemical	9.2	2.7	1.1	1.2	4.2
		Electrophoresis	9.1	3.1	0.7	1.1	1.2

TABLE II. COMPARISON OF PROTEIN FRACTIONATION BY CHEMICAL AND ELECTROPHORETIC METHODS\*

SERA	ALBUMIN	ALBUMIN PLUS		ALPHA GLOBULIN	BETA PLUS		
		ALPHA GLOBULIN	GLOBULIN		GAMMA	GLOBULIN	GLOBULIN
Human†	0.97	1.01	1.02	1.02	1.03	1.03	1.03
Farm animals‡	0.95	0.98	1.07	1.07	1.08	1.10	1.05

\*The results are expressed as the ratio of the protein fraction obtained by chemical methods to the protein fraction by electrophoresis.

†Results from one normal and three abnormal sera.

‡Results from three sheep, one horse, and one porcine sera.

## SUMMARY AND CONCLUSION

A precipitation method for the fractionation of sera is described. This method gives values for albumin and alpha, beta, and gamma globulin which show good agreement with those obtained by electrophoretic analyses.

## REFERENCES

1. Weichselbaum, T. E.: An Accurate and Rapid Method for the Determination of Proteins in Small Amounts of Blood Serum and Plasma, *Am. J. Clin. Path. (Techn. Sect.)* 10: 10, 1916.
2. Kingsley, G. R.: A Rapid Method for the Separation of Serum Albumin and Globulin, *J. Biol. Chem.* 133: 731, 1910.
3. Pillemer, L., and Hutchinson, M. C.: The Determination of the Albumin and Globulin Contents of Human Serum by Methanol Precipitation, *J. Biol. Chem.* 158: 299, 1915.
4. Cohn, E. J., McMeekin, T. L., Oncley, J. L., Newell, J. M., and Hughes, W. L.: Preparation and Properties of Serum and Plasma Proteins I. Size and Charge of Proteins Separating Upon Equilibration Across Membranes With Ammonium Sulfate Solutions of Controlled pH, Ionic Strength, and Temperature, *J. Am. Chem. Soc.* 62: 3386, 1910.
5. Ayer, J. B., Dailey, M. E., and Fremont-Smith, F.: Denis Ayer Method for the Quantitative Determination of Protein in the Cerebrospinal Fluid, *Arch. Neurol. & Psychiat.* 26: 1038, 1931.
6. Dole, V. P., and Braun, E.: The Electrophoretic Patterns of Normal Plasma, *J. Clin. Investigation* 23: 708, 1911.
7. Taylor, H. L., and Keys, A.: Fractionation of Normal Serum Proteins by the Electrophoretic and Sodium Sulfate Methods, *J. Biol. Chem.* 148: 379, 1943.
8. Petermann, M. L., Young, N. F., Hoagness, K. R., and Rhoads, C. P.: The Determination of Plasma Albumin by Chemical and Electrophoretic Methods, *Proc. Am. Federation Clin. Research* 3: 75, 1917.
9. Chow, Bacon F.: The Determination of Plasma or Serum Albumin by Means of a Precipitin Reaction, *J. Biol. Chem.* 167: 757, 1947.
10. Morison, Peter R.: On the Determination of Fibrinogen, *Federation Proc.* 6: 280, 1947.

# SODIUM AND POTASSIUM DETERMINATIONS IN HEALTH AND DISEASE

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THE data in this report will be presented in three segments: (1) the concentration of sodium and potassium in the sera of 107 normal adults, (2) the results of various recovery experiments, and (3) several cases demonstrating the clinical application of sodium and potassium determinations. The flame photometer<sup>1, 2</sup> offered a rapid means of accurately determining the concentrations of these ions.

## APPARATUS

The flame photometer used in these observations was the Perkin-Elmer instrument, Model 18. The recommended technique was followed.<sup>2</sup> The burner was fed natural gas from the city mains via the recommended constant pressure adjustment. The air pressure leading to the atomizer was maintained at 14 pounds. Two adjustments were necessary in relation to these features of the instrument before constant reproducible readings were obtained. By trial and error method the aperture of the jet giving the most constant readings was found to be 0.8 millimeter. This 0.8 mm. aperture was made by soldering a thin sheet of brass over the opening of the larger jet supplied and gradually reaming out a hole that would produce a flame of maximum heat without "feathering" (that is, loss of the small blue cones above the burner grill). Second, a moisture trap was placed in the air pressure line to keep chemically contaminated moisture away from the flame.

It is understood that improved instruments which circumvent these difficulties will be available in the future.<sup>3</sup>

## NORMAL SERUM SODIUM AND POTASSIUM VALUES

Serum Na and K concentrations were determined on 107 normal, ambulatory adults (blood donors). Most of these subjects had been at rest more than twenty minutes prior to the collection of samples. The samples were taken at the end of the blood collections in the blood bank and consisted of the last column of blood in the collecting tube. This blood was allowed to drain by gravity into a test tube after withdrawal of the needle from the vacuum bottle. Test tubes previously were rinsed scrupulously with sodium- and potassium-free distilled water. The clot was allowed to contract and the serum was obtained by centrifuging.

The actual analysis for sodium or potassium was simple. For Na, the serum was diluted 1:100 with distilled water. The instrument was set at zero

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with the same distilled water and at 100 with a standard sodium solution. The standard used contained 50 ppm of sodium in the form of pure NaCl. The sodium concentration was obtained by the equation  $C_u = \frac{C_s R_u}{R_s} \times 100$  where  $C_u$  is the concentration of the unknown,  $C_s$  the concentration of the standard,  $R_u$  the reading of the unknown, and  $R_s$  the reading of the standard. The equation is based on a linear relationship of dilutions of the 50 ppm standard with the instrument used (see Table VI). The K concentration determination was similar. The serum was diluted 1:10 with distilled water. The standard KCl solution contained 100 ppm of K.

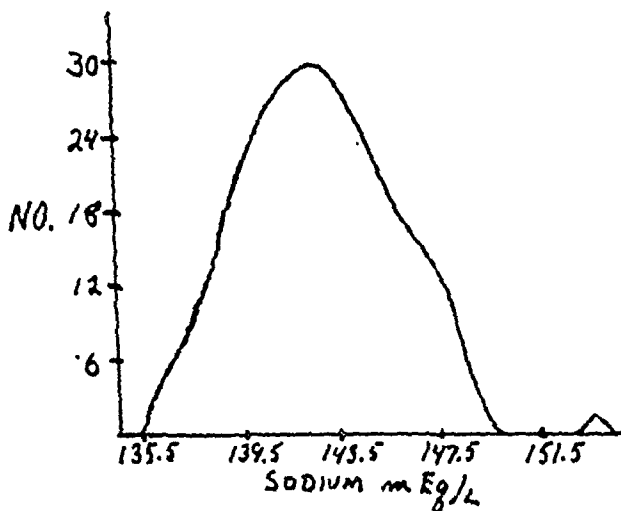


Fig. 1.—Mean, 141.0 meq. per liter; standard deviation, 3.6 meq. per liter.

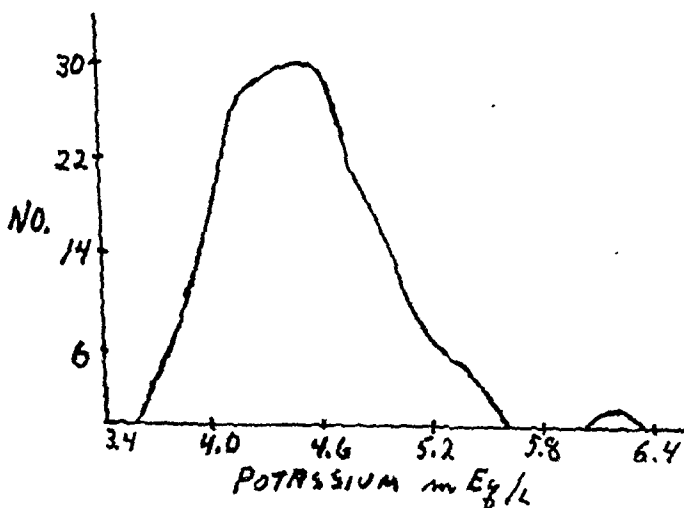


Fig. 2.—Mean, 4.52 meq. per liter; standard deviation, .45 meq. per liter.

Tables I and II contain the frequency distribution for the sodium and potassium determinations respectively. For the serum sodium values the following figures in milliequivalents per liter were determined: range 135.5 to

153.2; mean 144.0; standard deviation ( $\sigma$ ) 3.6; coefficient of variation, 2.5 per cent. The potassium values in milliequivalent per liter were: range 3.6  $\times$  6.2; mean 4.52; standard deviation 0.45; coefficient of variation 9.9 per cent. For comparison Table III shows the normal ranges as reported by other methods.

TABLE I. FREQUENCY DISTRIBUTION; NORMAL SERUM SODIUM DETERMINATIONS

NA (MEQ./L.)	NO.
135.5	1
137.5	9
139.5	22
141.5	29
143.5	30
147.5	13
149.5	1
151.5	0
153.5	2

TABLE II. FREQUENCY DISTRIBUTION; NORMAL SERUM POTASSIUM DETERMINATIONS

K (MEQ./L.)	NO.
3.6	1
3.9	11
4.2	28
4.5	30
4.8	20
5.1	9
5.4	5
5.7	0
6.0	1
6.2	1

TABLE III. NORMAL RANGES AS REPORTED BY OTHER METHODS

AUTHOR	NORMAL SERUM DETERMINA- TIONS NO.	RANGE		METHOD
		NA (MEQ./L.)	K (MEQ./L.)	
Kramer and Tisdall <sup>4</sup>	10		4.8-5.1	Unashed serum
	16		4.6-5.4	Ashed serum
Meyers and Short <sup>5</sup>	4		3.6-4.6	Ash method
Atchley, and others <sup>6</sup>	6		3.1-5.2	Precipitation method
	6	111.2-146.5		Gravimetric method
Rourke <sup>7</sup>	14	143.9-150.8		Iodometric method
Kramer and Tisdall <sup>8</sup>	17	140.4-152.1		Direct gravimetric
		145.0-150.0		Ashed
Consolazio and Talbott <sup>9</sup>	37	135.0-143.0	3.88-5.59	Electrodialysis
Consolazio and Talbott <sup>10</sup>	11		3.95-7.11	Titration
Sunderman <sup>11</sup>	10	133.6-137.3		Gravimetric method
	10		3.8-4.3	Titrimetric
Hald <sup>12</sup>	8	134.2-141.0	3.1-5.3	Dry ash method
Leva and Guest <sup>13</sup>	3	139.0-142.1	4.3-5.0	
Albanese and Wagner <sup>14</sup>	6		3.7-5.6	Colorimetric

## STANDARDIZATION OF ANALYTICAL PROCEDURES

Table IV contains data on estimations of sodium concentrations. Sixteen separate samples of serum from normal adults were divided into four groups

of four each. The following amounts of sodium in milliequivalent per liter were added: 2.17 to the first group, 10.85 to the second, 21.7 to the third, and 43.4 to the fourth. The fourth column in the table gives the expected theoretic amounts to be recovered, and the fifth column gives the actual quantities determined by the flame photometer. The deviations observed are given in Column six.

TABLE IV. RECOVERY OF SODIUM ADDED TO NORMAL HUMAN SERUM

SAMPLE	ORIGINAL VALUE (MEQ./L.)	NA ADDED (MEQ./L.)	THEORETIC NA CON- CENTRATION (MEQ./L.)	RECOVERY VALUE (MEQ./L.)	DEVIATION (MEQ./L.)
1	111.3	2.17	113.47	114.31	+0.87
2	115.7	2.17	117.87	119.31	+1.47
3	117.8	2.17	119.97	120.00	+0.03
4	150.9	2.17	153.07	150.00	-3.07
5	146.7	10.85	157.55	156.10	-1.45
6	143.0	10.85	153.85	153.90	+0.05
7	146.1	10.85	156.95	155.60	-1.35
8	141.6	10.85	152.45	157.80	+2.35
9	143.9	21.70	165.60	162.60	-3.00
10	146.1	21.70	167.80	166.90	-0.90
11	146.1	21.70	167.80	161.70	-3.10
12	145.7	21.70	167.40	166.10	-1.30
13	145.7	43.40	189.10	190.20	+1.10
14	145.2	43.40	188.60	186.10	-2.50
15	148.6	43.40	192.00	181.40	-7.60
16	149.3	43.40	192.70	193.00	+0.30

TABLE V. RECOVERY OF POTASSIUM ADDED TO NORMAL HUMAN SERUM

SAMPLE	ORIGINAL VALUE (MEQ./L.)	K ADDED (MEQ./L.)	THEORETIC K CON- CENTRATION (MEQ./L.)	RECOVERY VALUE (MEQ./L.)	DEVIATION (MEQ./L.)
1	4.90	1.92	6.82	7.00	+0.18
2	5.30	1.92	7.22	6.90	-0.32
3	5.00	1.92	6.92	7.50	+0.52
4	5.40	1.92	7.32	7.40	+0.08
5	5.00	3.84	8.84	9.20	+0.36
6	6.20	3.84	10.04	10.50	+0.46
7	4.60	3.84	8.44	8.70	+0.26
8	4.70	3.84	8.54	8.20	-0.34
9	4.90	7.68	12.58	12.30	-0.28
10	4.70	7.68	12.38	12.90	+0.52
11	6.30	7.68	13.98	13.50	-0.48
12	5.30	7.68	12.98	12.70	-0.28
13	4.60	11.52	16.12	15.80	-0.32
14	5.20	11.52	16.72	16.10	-0.62
15	4.50	11.52	16.02	15.60	-0.42
16	4.70	11.52	16.22	16.10	-0.12

Table V has identical information concerning the sixteen samples with reference to the potassium concentration in milliequivalent per liter.

The deviations observed in these determinations are considered to be within the variations of standard clinical laboratory tests and indicate a satisfactory clinical applicability of the procedure.

In the next group of observations variations in the concentration of the standard solutions were made and recovery was tested. With Na (Table VI)

a standard of 50 ppm was used. The instrument was set at 100 with this solution and dilutions of the solution were tested for recovery. Three different groups were tested. Table VII has the same type of information with K standard solutions containing 100 parts per million. The observations in these groups indicate a degree of recovery which is quite satisfactory for clinical use.

TABLE VI. PROPORTIONALITY OF PHOTOMETER READINGS TO VARIOUS CONCENTRATIONS OF SODIUM

GROUP	CONCENTRATION BY WEIGHT (PPM)	READING ON PHOTOMETER	RECOVERY (PPM)	DEVIATION (PPM)
I	50	100.0	50.0	0.0
	40	83.0	41.5	1.5
	30	64.8	32.4	2.4
	20	40.1	20.0	0.0
	10	22.3	11.1	1.1
II	50	100.0	50.0	0.0
	40	81.2	40.6	0.6
	30	63.2	31.6	1.6
	25	52.3	26.1	1.6
	20	43.0	21.5	1.5
	15	32.8	16.4	1.4
III	10	20.3	10.1	0.1
	50	100.0	50.0	0.0
	40	81.2	40.6	0.6
	30	60.3	30.1	0.1
	20	40.2	20.1	0.1
	10	20.6	10.3	0.3

TABLE VII. PROPORTIONALITY OF PHOTOMETER READINGS TO VARIOUS CONCENTRATIONS OF POTASSIUM

GROUP	CONCENTRATION BY WEIGHT (PPM)	READING ON PHOTOMETER	RECOVERY (PPM)	DEVIATION (PPM)
I	100	100.0	100.0	0.0
	75	75.2	75.2	0.2
	50	50.0	50.0	0.0
	25	24.8	24.8	0.2
	20	20.3	20.3	0.3
	15	15.1	15.1	0.1
	10	10.0	10.0	0.0
II	100	100.0	100.0	0.0
	75	74.7	74.7	0.3
	50	52.2	52.2	0.2
	25	24.9	24.9	0.1
	15	15.0	15.0	0.0
	5	4.9	4.9	0.1
III	100	100.0	100.0	0.0
	75	74.9	74.9	0.1
	50	50.3	50.3	0.3
	25	24.8	24.8	0.2
	20	20.0	20.0	0.0
	15	15.1	15.1	0.1
	10	10.1	10.1	0.1

The influence of the dilution of the serum on the concentration values was tested for Na and is related in Table VIII. Greater variations were observed in this group of observations than in the former group. The 1:100 dilution seemed preferable because of higher and more stable readings.

TABLE VIII. EFFECT OF DILUTION OF SERUM ON THE PROPORTIONALITY OF PHOTOMETER READINGS TO CONCENTRATIONS OF SODIUM

SERUM	1:100 DILUTION		0.5:100 DILUTION		DEVIATION
	READING	MLQ./L.	READING	MLQ./L.	MEQ./L.
I	63.2	137.4	31.1	135.2	2.2
II	74.0	160.9	37.8	161.3	3.1
III	70.2	152.6	35.1	153.9	1.3
IV	63.1	137.8	30.1	130.9	6.9
V	67.3	146.3	33.7	146.5	0.2

Next, duplicate samples were tested for their potassium concentration. The results, given in Table IX, were quite satisfactory.

## CLINICAL APPLICATION

The pertinent features of several cases are presented as illustrations of the applicability of this procedure to routine clinical use.

TABLE IX. CONSISTENCY OF DUPLICATE RESULTS

SAMPLE	READING	MEQ./L.	DEVIATION (MEQ./L.)
1A	13.1	3.3	
1B	13.3	3.4	0.1
2A	16.0	4.1	
2B	16.2	4.2	0.1
3A	17.5	4.5	
3B	17.1	4.4	0.1
4A	14.9	3.8	
4B	14.8	3.8	0.0
5A	16.7	4.3	
5B	16.7	4.3	0.0
6A	12.2	3.1	
6B	12.3	3.2	0.1
7A	13.3	3.4	
7B	13.6	3.5	0.1
8A	19.0	4.9	
8B	19.0	4.9	0.0
9A	17.4	4.5	
9B	17.2	4.4	0.1

TABLE X

DAY	BLOOD UREA (MG./100 C.C.)	SERUM NA (MEQ./L.)	SERUM K (MEQ./L.)	URINE VOLUME (C.C.)	URINE NA (GM.)	URINE K (GM.)
2	107	144	7.2	12		
3	186			30		
4	200	146	6.4	74		
5	245	137	5.1	39		
6	338			100		
7	458	135	5.4	124		
8	475	130	5.9	206		
9				540	1.4	0.16
10	406	135	4.6	1480	2.86	0.166
11	435			2300	4.59	0.62
12	458	132	5.0	6670	16.68	2.35
13	329	138	4.4	8770	19.4	2.2
14	279	129	5.1	6840	19.35	1.85
15	169	142	4.3	6425		
16	85.3			5950	8.45	0.67
17	76	145	4.4	3660		
18	64			6685		
19	53					

*Case 1*—The patient was D negative (Rh negative) and had been immunized to the D antigen by two pregnancies. After the second delivery she received 350 c.c. of D positive blood. A severe hemolytic reaction occurred and was followed by severe renal insufficiency (oliguria, heme casts, azotemia, hypertension, etc.). Hyponatremia developed but was prevented from becoming extreme by  $\text{NaHCO}_3$  therapy. The K concentration was elevated at first but dropped to normal levels later. Table X contains the data related to Na and K.

The average  $\text{NaHCO}_3$  intake per day prior to the diuresis was 4 grams. During the diuresis attempts were made to replace the Na and K lost on a gram per gram basis.

*Case 2*—The patient experienced extreme post partum bleeding which necessitated a hysterectomy. Hypotension existed for fifty one hours and was followed by renal insufficiency. A "force fluids" regime led to water intoxication. When seven eight days after the hypotension an alarmingly high serum potassium concentration was observed. Twenty grams of  $\text{NaHCO}_3$  were quickly given by stomach tube, followed by 130 c.c. of 5 per cent NaCl solution by vein in two doses. The K value receded from the danger level and the Na concentration was elevated. Table XI presents data on this case.

TABLE XI

DAY	BLOOD UREA (MG/100 C.C.)	SERUM NA (MEQ/L)	SERUM K (MEQ/L)	URINE VOLUME (C.C.)	URINE NA (GM.)	URINE K (GM.)
1				170		
2	146			375		
3				300		
4	236			515		
5				955		
6	221			1070		
7	215			910+		
8	299	118	10.5	1200	1.44	0.93
9	291	125	6.1	2200	1.6	
10				3000		
11	355	137	5.0	3700		
12	370	137	4.1	5000	9.7	3.3
13	330	155	2.5	1300		

This patient died on the fourteenth day and an autopsy demonstrated severe and generalized brain damage. The kidneys were recovering satisfactorily at the time of death. The patient was in fluid balance except for a lowered K concentration which was being corrected.

*Case 3*—This patient had a K deficit following a partial gastrectomy with posterior gastrojejunostomy. There was a poorly functioning stoma and vomiting was prolonged. He had received dextrose solution by vein daily. He was treated with KCl\*. The diag

TABLE XII

POSTOPERATIVE DAY	SERUM K (MEQ/L)	SERUM NA (MEQ/L)	THERAPY AND COURSE
12	3		Muscular weakness, hyperactive reflexes, irrational
14	3.6	146.0	Clinical improvement after 40 cc 15% KCl I.V.
15	3.8		25 cc 15% KCl I.V.
16	3.9		20 cc 15% KCl I.V.
17	4.2		Stoma functioning, considered in fluid balance
19	4.3		
20	4.5		
21	4.5		
23	4.6		Four days previous to hospital discharge

\*By Dr. Louis Kregel (Resident in Surgery) and Dr. Carl Moyer (Consultant).

noses\* were (1) extracellular volume deficit with alkalosis and relative water excess and (2) relatively severe potassium deficit. The K values are shown in Table XII.

*Case 4.*—This was a case of fluid imbalance following a partial gastrectomy with Polya-Balfour anastomosis. On the seventh post-operative day a diagnosis of extracellular fluid deficit with a relative water excess was made by the surgical staff. Frequent serum Na and K determinations along with the clinical signs facilitated the handling of this case with hypertonic NaCl solution. The data are shown in Table XIII.

TABLE XIII

POSTOPERATIVE DAY	SERUM NA (MEQ./L.)	SERUM K (MEQ./L.)	THERAPY AND COURSE
6	123	7.7	
7	129.0	6.6	200 c.c. 5% NaCl I.V.
8	137	5.6	400 c.c. 5% NaCl I.V.
10	152	5.1	
12	137	5.6	Clinically in fluid balance

TABLE XIV

HOSPITAL DAY	SERUM NA	SERUM K (MEQ./L.)	24 HOUR GASTRIC ASPIRATIONS (GM.)		24 HOUR URINE (GM.)	
			NA	K	NA	K
12	149.6	3.6				
14			.293	.039	.975	.510
18	156.5	2.7	1.287	.586	.881	.593
19			.616	.158	1.350	.549
20			.408	.094	1.996	.740
21			1.105	.119	2.130	1.360
24			1.849	.591	2.820	1.670
25			1.510	.764	1.986	1.221
26			1.330	.683	2.073	1.170
38	146	4.2				

TABLE XV

DAY	SERUM NA (MEQ./L.)	SERUM K (MEQ./L.)
1	115.7	6.7
2	126.5	5.4
3	135.0	

TABLE XVI

DAY	SERUM NA (MEQ./L.)	SERUM K (MEQ./L.)
6	148	4.2
13	135	5.1
15	141	4.1
18	139	4.4

*Case 5.*—This case was one of chronic pyloric obstruction secondary to duodenal ulcer. There were Na and K determinations of the serum, gastric contents, and urine. There was a persistently low serum K concentration without any clinical signs of K deficit. After three weeks a gastroenterostomy was performed; recovery was uneventful. Table XIV presents the data.

\*Made by Dr. Moyer.

*Case 6.*—A patient with Addison's disease was studied during the terminal crisis. The values of K and Na during the last three days are shown in Table XV.

*Case 7.*—A patient with adenocarcinoma of the stomach was studied during the post-operative period. The normal values obtained are shown in Table XVI.

#### SUMMARY

1. A series of observations of the concentration of Na and K in body fluids has been made using the flame photometer technique.

2. Determination of sodium and potassium in the blood sera of 107 normal blood donors was made. The average for sodium was 144.0 meq. per liter, with a range of 135.5 to 153.2 meq. per liter. The average for potassium was 4.52 meq. per liter, with a range of 3.6 to 6.2 meq. per liter.

3. Recovery experiments, duplicate determinations, and distribution curves of normal values indicate satisfactory accuracy for clinical use.

4. The presence of sodium, and also potassium, in the blood sera of several patients with various diseases was determined.

#### REFERENCES

1. Barnes, R. B., Richardson, D., Berry, J. W., and Hood, R. L.: *Flame Photometer: A Rapid Analytical Procedure*, Indust. & Engin. Chem. (Anal. Ed.) 17: 605, 1945.
2. Brochure, The Perkin-Elmer Corp., 1945.
3. Hald, Pauline M.: *The Flame Photometer for the Measurement of Sodium and Potassium in Biological Materials*, J. Biol. Chem. 167: 499-510, 1947.
4. Kramer, Benjamin, and Tisdall, Fredrick, F.: *A Clinical Method for the Quantitative Determination of Potassium in Small Amounts of Serum*, J. Biol. Chem. 46: 339-349, 1921.
5. Meyers, Victor C., and Short, James J.: *The Potassium Content of Normal and Some Pathological Human Bloods*, J. Biol. Chem. 48: 83, 1921.
6. Atchley, Dana W., Loeb, Robert F., Benedict, Ethel, and Palmer, Walter W.: *Physical and Chemical Studies of Human Blood Serum*, Arch. Int. Med. 31: 606, 1923.
7. Rourke, Dorothy: *On the Determination of the Sodium Content of Small Amounts of Serum or Heparinized Plasma by Iodometric Method*, J. Biol. Chem. 78: 337, 1928.
8. Kramer, Benjamin, and Tisdall, Fredrick F.: *A Simple Method for the Direct Quantitative Determination of Sodium in Small Amounts of Serum*, J. Biol. Chem. 46: 467-473, 1921.
9. Consolazio, W. V., and Talbott, John H.: *The Determination of Total Base in Biological Material by Electrodeialysis*, J. Biol. Chem. 132: 753, 1940.
10. Consolazio, W. V., and Talbott, John H.: *Modification of the Method of Shohl and Bennett for the Determination of Potassium in Serum and Urine*, J. Biol. Chem. 126: 55-61, 1938.
11. Sunderman, William F.: *Studies of Serum Electrolytes: VII. The Total Base and Protein Components of the Serum During Lobar Pneumonia With a Note on Gastric Secretion*, J. Clin. Investigation 9: 615-633, 1930.
12. Hald, Pauline M.: *Notes on the Determination and Distribution of Sodium and Potassium in Cells and Serum of Normal Human Blood*, J. Biol. Chem. 163: 429, 1946.
13. Leva, E., and Guest, G. M.: *A Method for the Determination of Total Base in Blood*, J. Biol. Chem. 130: 777, 1939.
14. Albanese, Anthony A., and Wagner, Dorothy L.: *A Method for the Colorimetric Determination of Potassium in Biologic Products*, J. LAB. & CLIN. MED. 30: 280-284, 1945.



# CHANGES IN THE BONE MARROW IN MEGALOBLASTIC ANEMIAS OF INFANCY BEFORE AND AFTER FOLIC ACID THERAPY

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UNDER the term megaloblastic anemia of infancy we have previously described an anemia characterized by changes in the blood and bone marrow resembling those of Addisonian pernicious anemia and by a specific response to folic acid.<sup>1-3</sup> The present report deals with the results of serial studies of the bone marrow which were undertaken in patients with this type of anemia for the purpose of elucidating the mechanisms underlying the development of the megaloblastic dysplasia and its disappearance under specific therapy.

## MATERIAL AND METHODS

Our material consisted of fifteen patients\* with megaloblastic anemia observed during a period of twenty one months at the Children's Hospital of Michigan. All the patients were white infants ranging in age from five weeks to one year. The clinical features were not characteristic. Usually there were signs of infection, and in three instances scurvy was noted. Eleven of the fifteen patients had histamine resistant achlorhydria at the time therapy was begun. Macrocytic anemia of varying degree was present in all cases. The hemoglobin levels on admission ranged from 1.7 to 8.3 Gm. per 100 c.c. (Table I). The percentage of reticulocytes was variable and in some instances moderately elevated even before therapy. Prior to folic acid therapy six patients received transfusions of whole blood and two others, transfusions of saline suspended washed red cells. Folic acid† was administered by intramuscular injection in all but two instances, the daily dosage varying from 10 to 100 mg. and the total dosage, from 50 to 900 milligrams. The average course of treatment consisted of two daily injections of 10 mg. over a period of one week. To two patients folic acid (*Lactobacillus casei* factor) was given by mouth in daily doses of 5 mg. for ten day periods. Since it was evident that there was no appreciable difference in the response between patients receiving lower and higher dosage within the range given, no further reference to dosage will be made. In every case a characteristic increase in reticulocytes, up to 23 per cent, took place around the fifth day after the start of therapy, regardless of the initial degree of reticulocytosis, and hemoglobin and red cell counts rose significantly.

Daily complete blood counts and reticulocyte counts were made. Bone marrow was aspirated from the femur, and occasionally from the upper end of the tibia. In each instance at least one marrow sample before and after treatment was taken and in some instances as many as six serial examinations were made. A measured amount of marrow was withdrawn, usually between 1.0 and 2.0 c.c., although occasionally more or less marrow was accidentally obtained. The samples were placed in paraffin tubes containing heparin. One cubic centimeter was transferred into a Wintrobe tube, centrifuged for five minutes at 3,000 r.p.m., and the height of the various layers was recorded. The myeloid erythroid

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\*Clinical data on nine of these patients were included in our earlier reports<sup>2, 3</sup>.

†Synthetic folic acid was obtained through the courtesy of Lederle Laboratories, Inc., Pearl River, N. Y.

TABLE I. BLOOD, BONE MARROW, AND GASTRIC JUICE BEFORE THERAPY

CASE	INITIAL BLOOD FINDINGS			INITIAL MARROW FINDINGS			PRESENCE OF FREE HCL AFTER HISTAMINE
	HEMO- GLOBIN (GM./ 100 C.C.)	R.B.C. (MIL./ C.MM.)	RETICULO- CYTES (%)	MARROW ASPIRATED (C.C.)	MYELOID- ERYTHROID LAYER (VOL. %)	ERYTHROID LAYER* (VOL. %)	
1	5.0	1.12	1.5	1.0	2.0	0.7	0
2	1.9	1.19	6.9	1.3	1.0	2.0	0
3	6.8†	2.52	0.9	2.25	20.0	9.0	+
4	8.0	2.25	3.1	1.5	30.0	11.1	0
5	3.5	1.12	0.1	?	2.0	1.0	0
6	1.7	0.30	0.3	0.7	0.91	0.006	0
7	5.8	2.01	0.7	1.5†	10.5	1.5	0
8	8.3	2.53	2.7	1.1	12.0	3.2	+
9	6.6	2.05	2.7	2.2	5.0	1.8	0
10	7.7	2.75	5.8	2.0	26.0	13.9	+
11	5.0	1.33	1.3	2.25	2.0	0.8	0
12	6.2	2.40	1.0	2.25	5.0	1.0	†
13	7.8	2.30	0.6	2.0	15.0	4.1	0
14	4.2	0.89	1.2	3.0	3.6	0.9	+
15	6.2	2.11	8.7	1.1	7.5	3.3	0

\*Calculated from the proportion of erythroblasts obtained in a differential count of 500 nucleated cells.

†After transfusion.

‡Not examined.

layer was then thoroughly mixed on a paraffin-coated watchglass and films made from it were stained with May-Gruenwald-Giemsa. In each case a differential count of at least 500 nucleated cells was made, using the nomenclature of Downey<sup>4</sup> and Jones.<sup>5</sup> In addition, a count of 500 erythroblasts was made for each aspiration in eleven instances and in eight of these 100 mitoses in erythroblasts were classified for every sample.

#### QUALITATIVE CHANGES

*Changes Before Therapy.*—In six of the fifteen patients erythropoiesis was frankly megaloblastic.\* As a rule the proportion of basophilic cells was high. This "shift to the left" in the maturation curve of the erythroid cells was paralleled by a relative increase in the number of mitoses encountered in basophilic cells. In examining many fields the total number of mitoses rarely appeared significantly increased and in some instances was clearly depressed, but actual counts were not made because the incidence of mitotic figures in only 500 cells would not have been significant. The mitotic figures were often distinctly pathologic. The chromosomes frequently appeared abnormally long and slender, the polarity of the mitotic spindle was often distorted, and aberrant chromosomes were common. Karyorrhexis and karyolysis were often pronounced. The granulocytes showed alterations in size, nuclear configuration, and cytoplasm indistinguishable from those seen in pernicious anemia.

In seven other patients classic megaloblasts were also present in fair numbers but did not constitute the majority of the erythroid cells. The predominant cell type, although closely resembling the megaloblast, failed to meet the strict criteria given by Jones<sup>5</sup> and others, yet these erythroblasts were distinctly abnormal and different from normoblasts. In the course of our studies it soon became evident that the current nomenclature of marrow cells which implies two types of erythropoiesis was inadequate for our purpose. It would

\*For illustrations see our earlier communication.<sup>2</sup>

have forced us to place pathologic cells in the category of normoblasts simply because they did not correspond in every detail to megaloblasts. We therefore temporarily adopted the term "intermediate" for erythroid cells which we could not otherwise classify. We do not wish to imply that these cells represent a separate type of erythropoiesis but merely to indicate that *morphologically* they occupy a middle position between typical normoblasts and typical megaloblasts.

The cells of the intermediate type were large, usually as large as megaloblasts and always significantly larger than normoblasts. The ratio of cytoplasm to nucleus was often increased. The nucleus had an increased amount of acidophilic matter and the chromatin net was much finer and more regular than that of the normoblast but still slightly coarser than that of the classic megaloblast. The open structure of the nuclear chromatin persisted in the later, acidophilic stages of cell development, and in the more mature forms differentiation from megaloblasts became indeed almost impossible. As in typical megaloblasts, abnormal mitoses, irregular chromatolysis, and pronounced karyorrhexis were common features of these intermediate cells, and many of the nuclei, especially those of the later stages, had bizarre shapes and showed extreme lobulation.

We concluded that this intermediate type of erythropoiesis represents an early stage of megaloblastic transformation of the bone marrow. We were led to this view by the following observations:

1. The cells designated as intermediate resembled megaloblasts in all but the finest cytologic details.

2. At least a few, and often numerous, classic megaloblasts were always found when erythropoiesis was of the intermediate type but were never seen by us in normoblastic marrows. Conversely intermediate cells were nearly always present when megaloblasts predominated in the marrow.

3. The actual progression of megaloblastic transformation was observed in one patient (Fig. 1) where specific treatment was deliberately withheld for one month after the initial marrow examination had shown an intermediate type of erythropoiesis. At the end of that time the anemia had recurred and a second marrow specimen showed a considerable increase in classic megaloblasts.

4. In every case where intermediate erythroblasts predominated in the marrow the abnormalities seen in the granulocytes were the same as in cases with a frankly megaloblastic picture. We have not seen this type of leucopoiesis in other conditions.

5. A specific effect of folic acid was demonstrable in every instance and, as will be shown the response followed the same pattern as that of the first group (Fig. 7).

These observations suggested that the megaloblastic transformation of the bone marrow is a gradual and progressive change in which the appearance of typical megaloblasts and promegaloblasts is a relatively late event. Our findings seem to indicate that the process may be recognized in its earlier stages by the application of more liberal criteria in regard to erythropoiesis. Even

■ MEGALOBLASTS  
 ▨ INTERMEDIATE  
 □ NORMOBLASTS

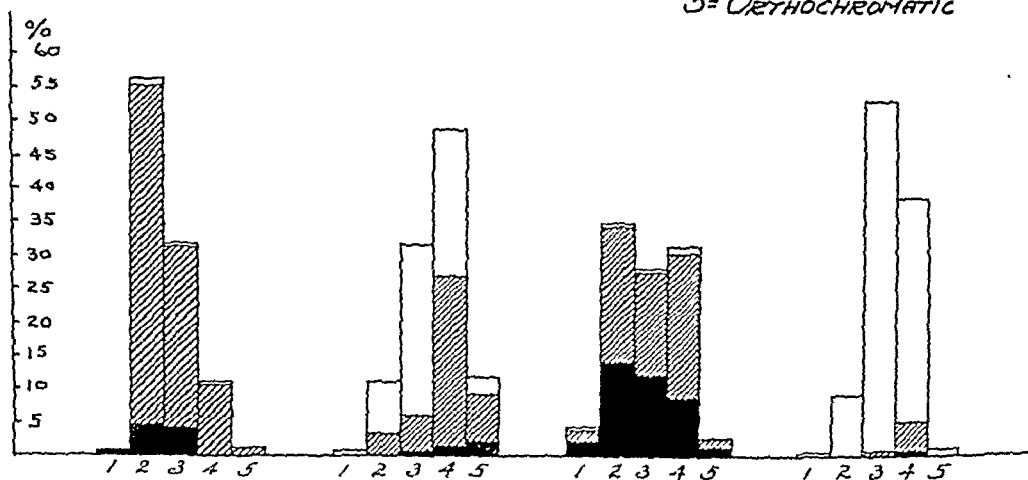
1 = PROERYTHROBLASTS

2 = BASOPHILIC ERYTHROBLASTS

3 = POLYCHROMATOPHILIC

4 = OXYPHILIC

5 = ORTHOCHROMATIC



1st Admission

9-11-46

After Transfusion & Washed R.B.C.

9-18-46

10-23-46

10-25-46

Fig. 1.—Case 13. The change in marrow from intermediate to megaloblastic type when specific therapy is withheld.

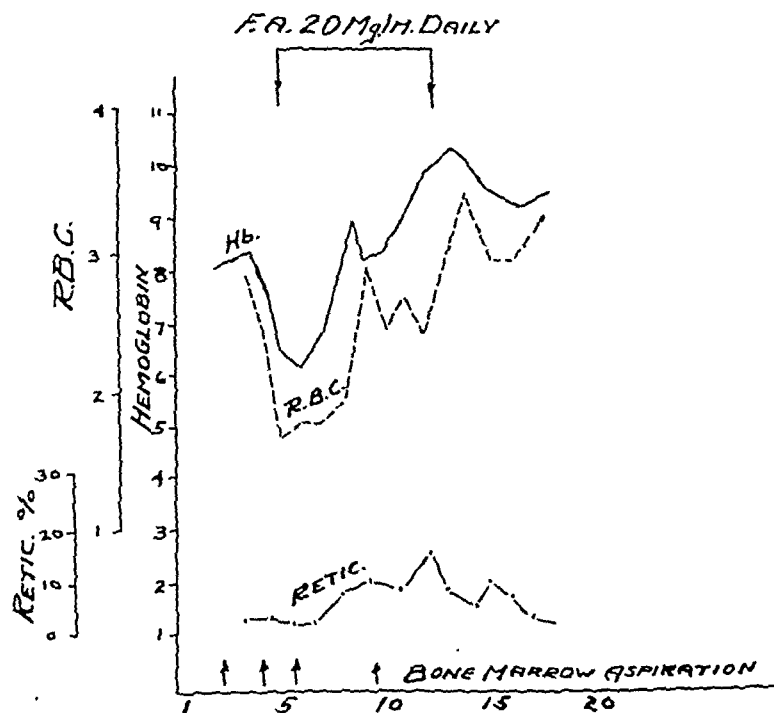


Fig. 2.—Case 1. Response of anemia to folic acid.

the pattern of erythropoiesis which we have referred to as intermediate appears to be preceded by constant and specific changes in the granulocytes which may be utilized for early diagnosis. The marrow of the remaining two patients in our group (Cases 4 and 7) showed only minimal deviations from the normoblastic pattern, but the normal myelocytes were largely replaced by "giant metamyelocytes" and similar pathologic granulocytes. In both instances the administration of folic acid was followed by reticulocyte responses of specific character and by subsequent rises in hemoglobin and red cell counts without further treatment (Fig. 2). Similar effects of folic acid were not seen in anemias other than those associated with megaloblastic bone marrow.<sup>3</sup>

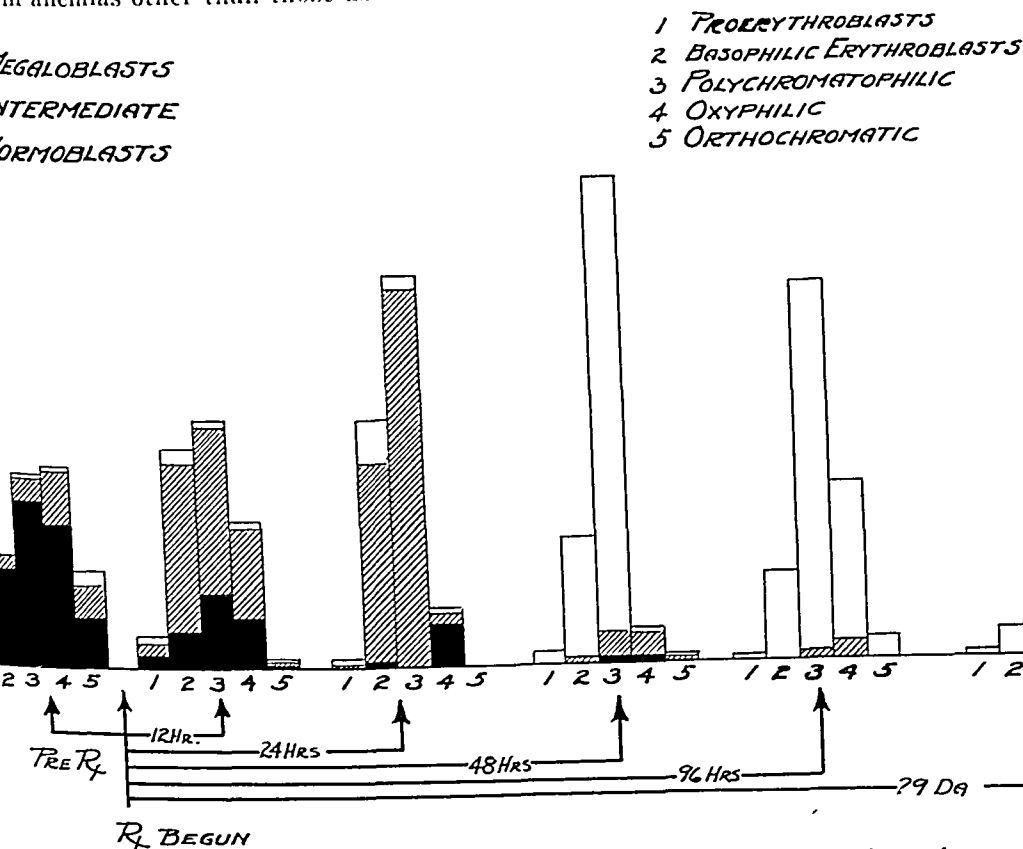


Fig. 3.—Case 8. Qualitative changes in erythropoiesis in response to folic acid.

*Changes After Therapy.*—In studying the marrow response to folic acid an attempt was made to obtain an over-all picture by taking samples at different intervals after the start of therapy. Therefore, the specimens taken from different patients were not always comparable as to the time when they were taken, but ranged from twelve hours to twenty-nine days, with most of the samples obtained in the first five days after treatment. Since it soon became evident that most of the significant changes took place during that period only a few observations were made in later stages.

Our only twelve-hour specimen came from a patient with a characteristic megaloblastic marrow pattern (Fig. 3). Before treatment almost no normo-

blastic cells were present. After twelve hours a significant change had occurred. Megaloblasts were still present but the vast majority of the cells had undergone a distinct change in appearance. Again, since they could not otherwise be classified, these cells were designated as intermediate in type. A small number of basophilic normoblasts was noted at this time.

At twenty-four hours the marrow of the same patient showed few megaloblasts and virtually all of these were acidophilic cells. There was a marked increase in basophilic and polychromatic erythroblasts which still did not appear entirely normal but closely resembled normoblasts. Simultaneously an appreciable number of basophilic normoblasts appeared. Numerous mitotic figures were seen.

At forty-eight hours erythropoiesis was typically normoblastic. There was a further marked increase in polychromatic erythroblasts.

At ninety-six hours a shift toward more mature cells had become evident. Almost 30 per cent of the erythroblasts were cells with oxyphilic cytoplasm.

Serial studies made on nine other patients showed a similar pattern (Figs. 4 to 7).<sup>\*</sup> On the basis of these composite observations the progression of marrow changes in response to folic acid may be summarized as follows: The megaloblasts lose their characteristic structure, and in twelve to twenty-four hours the erythroblasts assume an appearance intermediate between that of megaloblasts and normoblasts. Typical normoblasts can already be seen in small numbers. Almost the only megaloblasts still recognizable at this time are late, oxyphilic cells, and these disappear at the end of thirty-six hours. Two days after treatment has begun the transformation is virtually complete and the marrow is normoblastic.

The change in the cellular characteristics is accompanied by an increase of basophilic and, slightly later, of polychromatic cells and by the appearance of numerous mitotic figures. In the following days there is a relative decrease in basophilic erythroblasts, a further increase in polychromatic cells, and a steady increase in oxyphilic cells so that the distribution of erythroid cells gradually approaches the normal.

The shifts in the maturation curves of the erythroblasts were rather closely paralleled by shifts in the distribution of the mitoses (Fig. 8). Before treatment there was usually a relative increase in mitosis of basophilic cells and a corresponding decrease of mitosis in more mature erythroblasts. Following therapy an impressive increase in the proportion of polychromatophilic cells in mitosis took place (Fig. 9). This suggested that the earlier disturbance had affected mitotic activity chiefly at the level of the polychromatophilic erythroblasts and that one effect of specific therapy was the resumption of mitotic division at the normal level of cellular maturation.

Our findings indicate that only the late, oxyphilic megaloblasts escape the effect of folic acid and are not transformed into normoblasts, presumably because they are past the stage in cellular development where multiplication occurs. These cells continue their maturation and probably leave the marrow

<sup>\*</sup>To save space additional graphs showing the pattern in the four remaining cases have been omitted since they have essentially the same features as those shown.

- MEGALOBLASTS  
 ▨ INTERMEDIATE  
 □ NORMOBLASTS
- 1 PROERYTHROBLASTS  
 2 BASOPHILIC ERYTHROBLASTS  
 3 POLYCHROMATOPHILIC  
 4 OXYPHILIC  
 5 ORTHOCHROMATIC

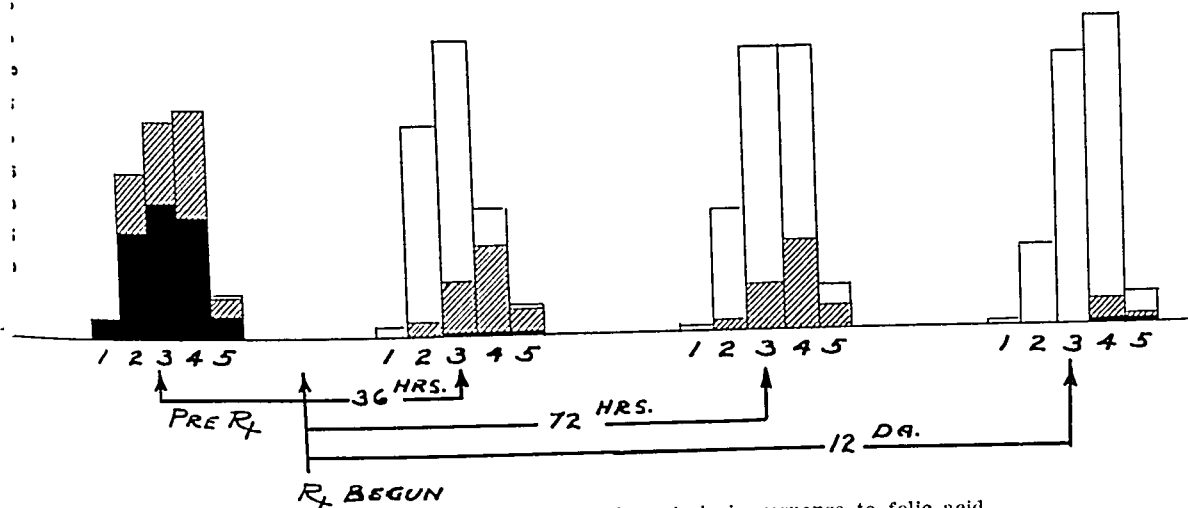


Fig. 4.—Case 15. Qualitative changes in erythropoiesis in response to folic acid.

- MEGALOBLASTS  
 ▨ INTERMEDIATE  
 □ NORMOBLASTS

- 1 = PROERYTHROBLASTS  
 2 = BASOPHILIC ERYTHROBLASTS  
 3 = POLYCHROMATOPHILIC  
 4 = OXYPHILIC  
 5 = ORTHOCHROMATIC

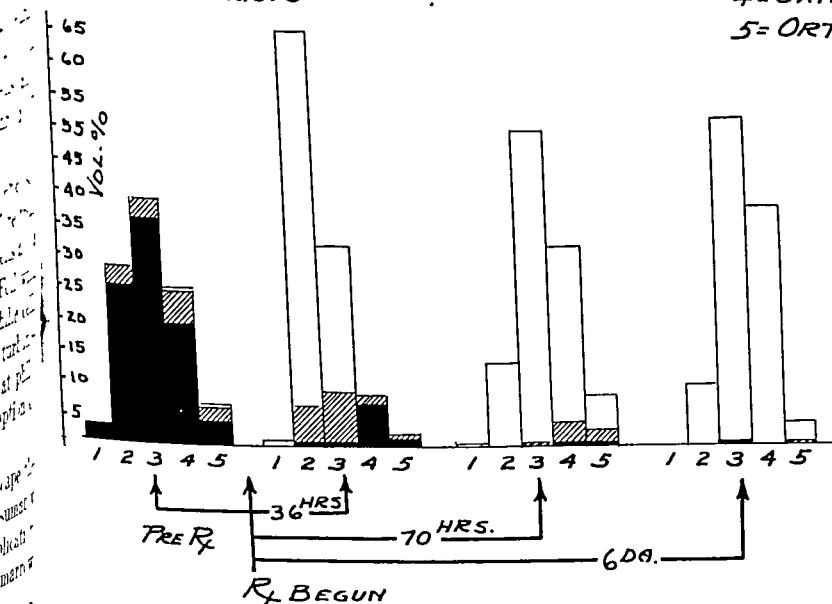


Fig. 5.—Case 6. Qualitative changes in erythropoiesis in response to folic acid.

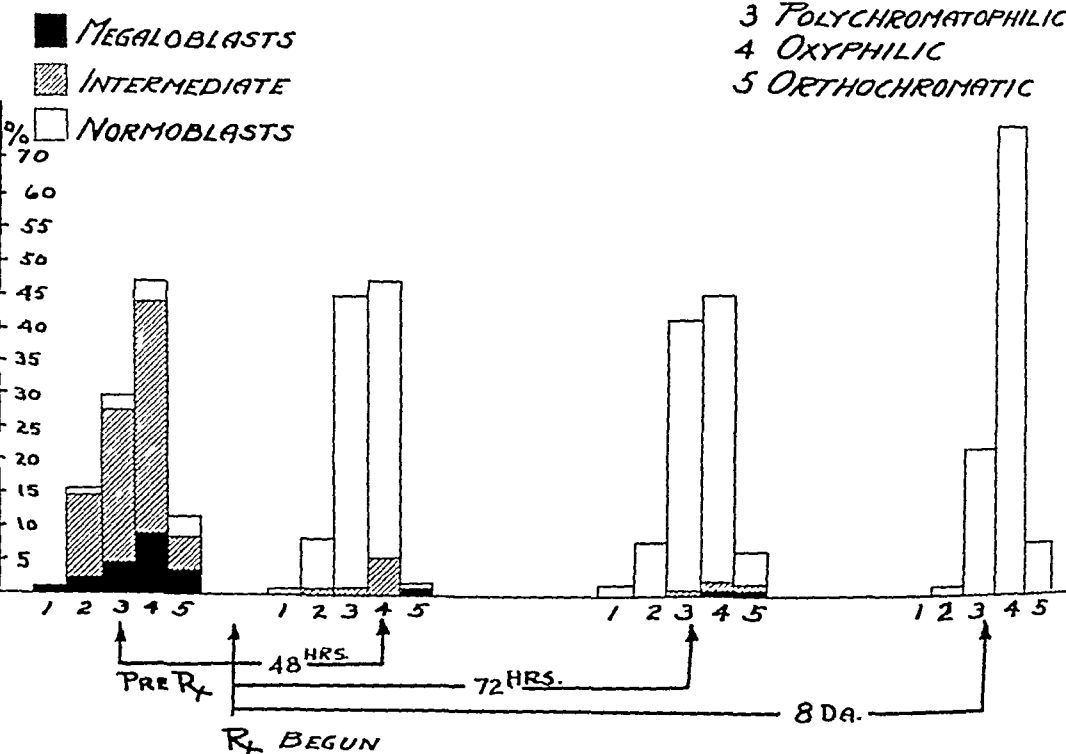


Fig. 6.—Case 9. Qualitative changes in erythropoiesis in response to folic acid.

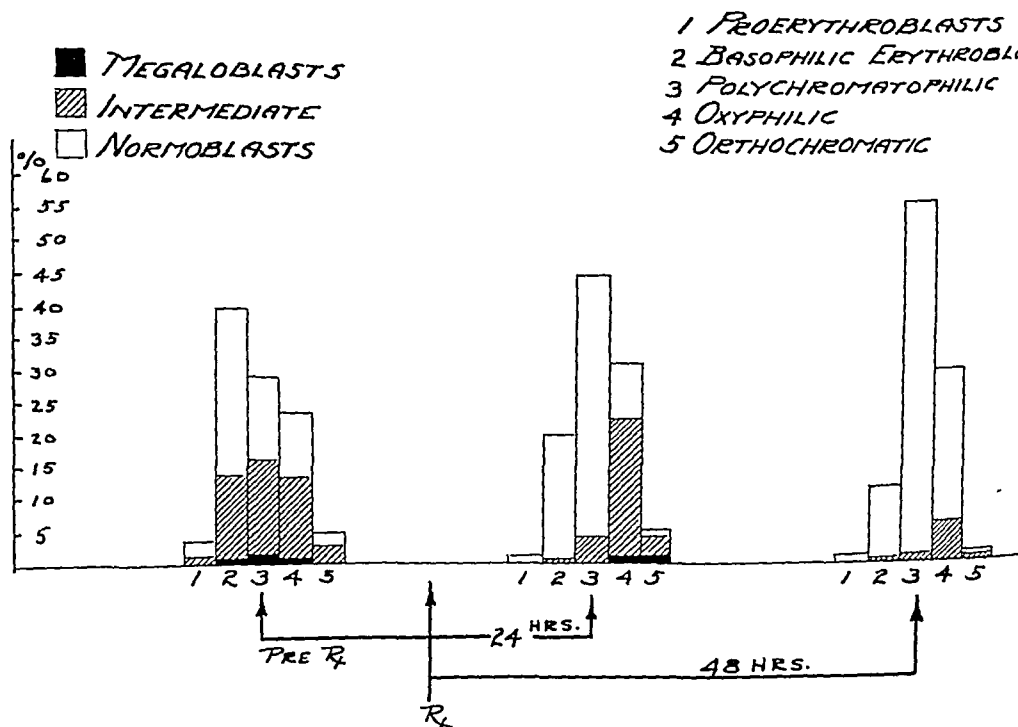


Fig. 7.—Case 5. Qualitative changes in erythropoiesis in response to folic acid.



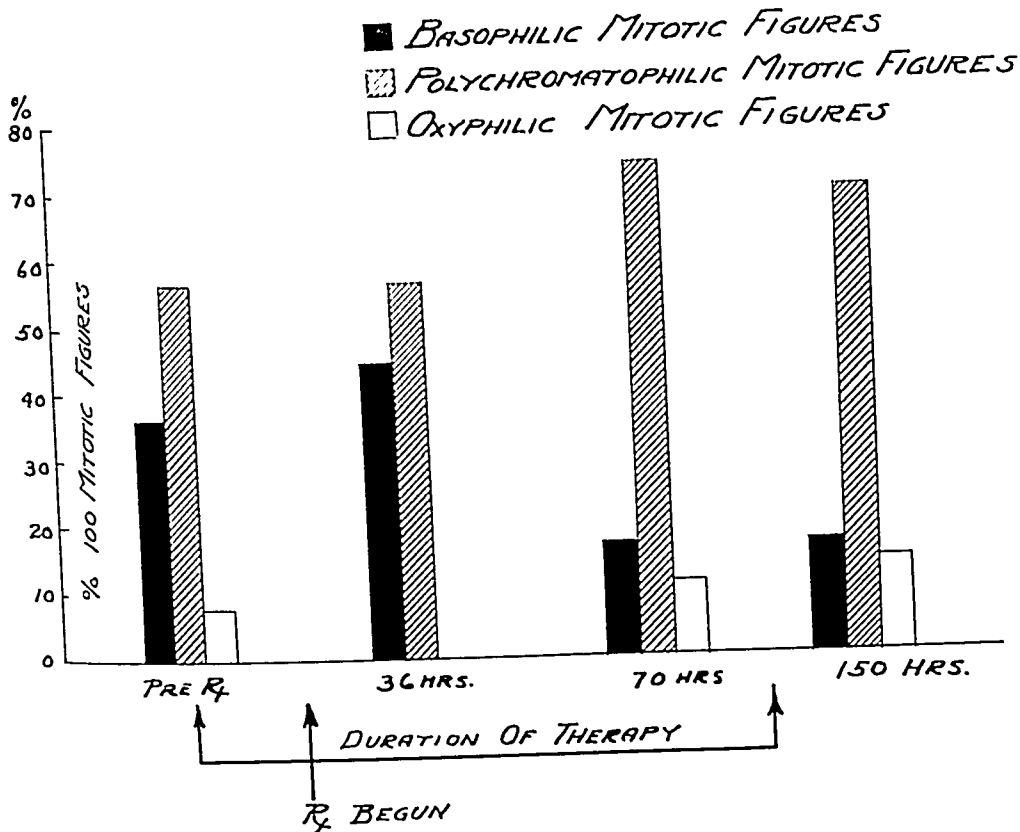


Fig. 8.—Case 6. Shift in erythroid mitosis after therapy.

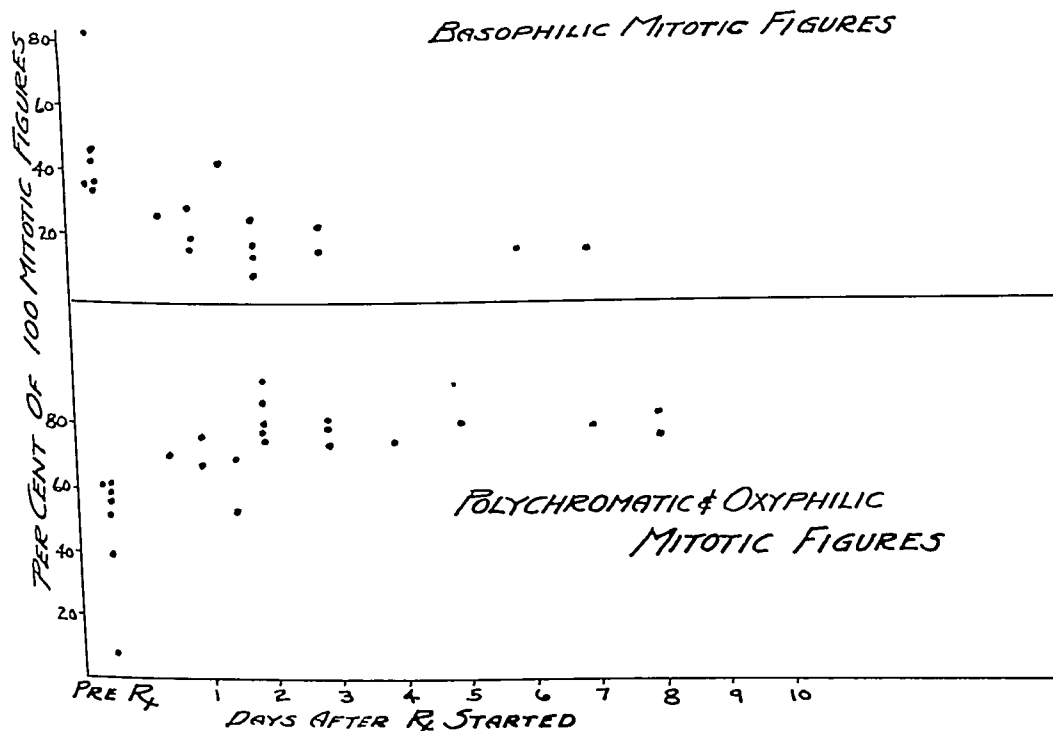


Fig. 9.—Trends of erythroid mitosis with therapy in eight cases.

as megalocytes. The younger megaloblasts, on the other hand, seem to be transformed almost quantitatively into normoblasts, even those already in the polychromatic stage. This is indicated not only by the enormous increase in polychromatic normoblasts early in the course of the response, an event which could hardly be explained by proliferation of the few residual normoblasts present before treatment, but more significantly by the virtually complete disappearance of megaloblasts in twenty-four to thirty-six hours. If the younger megaloblasts were not affected by the therapy but continued to mature as megaloblasts they should be demonstrable in proportionate numbers as oxyphilic megaloblasts in subsequent marrow samples. It appears likely, therefore, that as late as the polychromatic stage megaloblasts can utilize the hemopoietic principle and resume normal mitosis and normal morphologic characteristics.

Our observations on the evolution and regression of the megaloblastic pattern support neither the view that megaloblasts are normal constituents of the marrow nor the concept that these cells are genetically different from normal erythroblasts and immutably fixed in their characteristics by the nature of their ancestral cells. Instead we interpret our findings as evidence that in the absence of hemopoietic principle erythroblasts undergo a progressive aberration in structure as well as function which is reversible except in cells permanently incapable of mitosis. This concept in no way detracts from the diagnostic significance of the megaloblast.

## QUANTITATIVE CHANGES

We are well aware of the limitations of measurements in the cellularity of the bone marrow.<sup>6</sup> Nevertheless it seemed worth while to investigate the volumetric pattern of the aspirated marrow in our patients to see if a constant pattern was demonstrable. In regard to the total cellularity before therapy this was not the case. Table I shows that the height of the myeloid-erythroid layer in the initial samples was too variable to be significant in itself. However,

TABLE II. CHANGES IN THE MYELOID-ERYTHROID LAYER WITH FOLIC ACID THERAPY

[illegible]

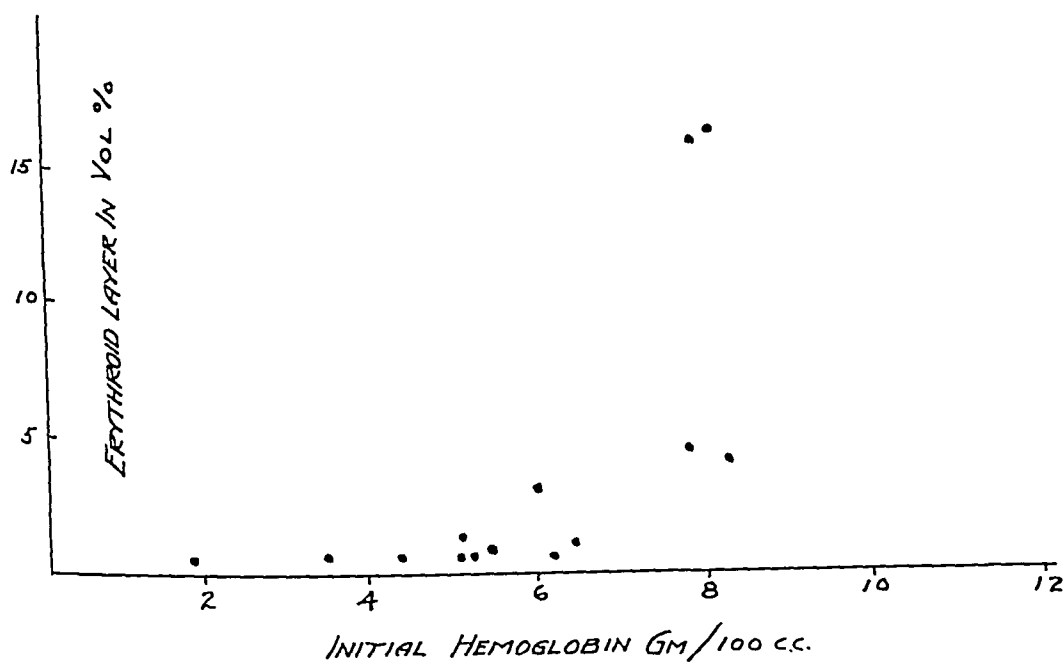


Fig. 10.—Relation of erythroid layer of initial marrow to initial hemoglobin.

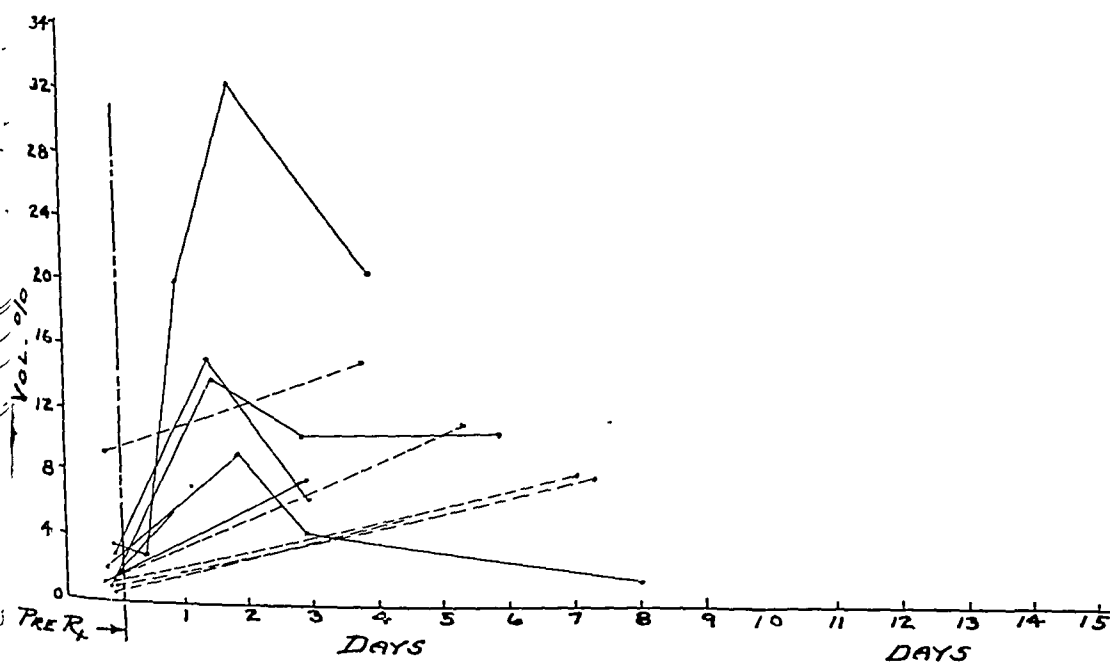


Fig. 11.—Increase in erythroid layer of buffy coat after folic acid therapy in twelve of fifteen cases. Dotted lines indicate only one marrow sample taken after treatment.

a substantial rise in the myeloid-erythroid volume was observed after therapy in eleven of the fifteen patients, suggesting a significant trend (Table II).

When the height of the myeloid-erythroid layer after centrifugation was recorded in volumes per cent and the proportion of erythroblasts determined by differential counts of 500 nucleated cells, the volume of erythroblasts could be expressed in per cent of the total sample, giving a sample measure of erythropoietic activity. Using these values we found rather good correlation between the initial erythroid cell volume in the marrow and the severity of the anemia (Fig. 10). Surprisingly enough, erythroid hyperplasia was the more marked the higher the hemoglobin; it was slight or absent in patients

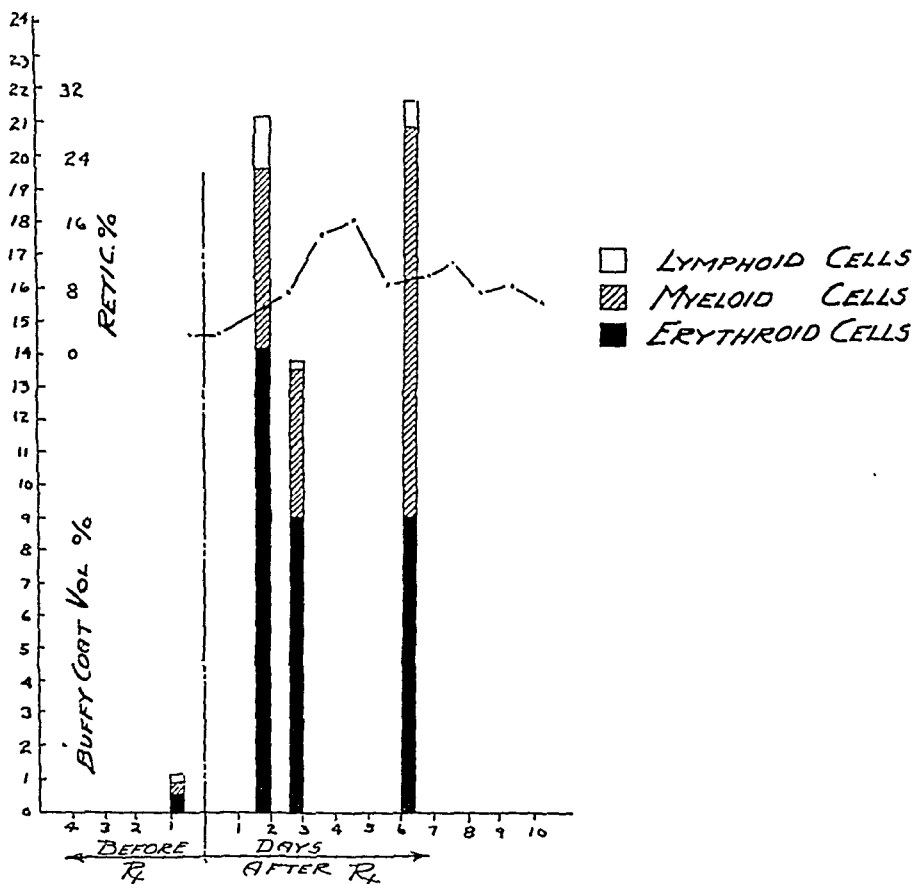


Fig. 12.—Case 6. The effect of folic acid on the height and constituents of the myeloid-erythroid layers.

with severe anemia. This behavior of the erythroid marrow cells is in contrast to that of uncomplicated pernicious anemia in the adult<sup>7</sup> and suggests a non-specific depression of erythropoiesis probably related to the presence of active infection superimposed on the deficiency of hemopoietic principle.

When the erythroid cell volume alone was used as a measure of the response to therapy, a clear-cut increase was demonstrable in twelve of the fifteen cases (Fig. 11), a decrease in two (Cases 4 and 10), and no appreciable

change in one (Case 13). This trend indicates that, for the purpose of a comparative study at least, the method used by us is valid. Using this measure it became evident that in two of the four subjects in whom a drop in the total myeloid-erythroid layer occurred after therapy the decline was not due to a decrease in erythroblasts but to a decrease of other marrow cells (granulocytes and lymphocytes). In one of these subjects (Case 7) the erythroid cells actually increased significantly in spite of the total decrease in myeloid-erythroid volume.

In some instances the rise in the volume of erythroid cells was tremendous, amounting to a thirty fold increase in twenty-four hours in one patient (Fig. 12). Of the two instances in which a drop occurred, one was in a patient (Case 10) whose blood showed reticulocytosis of 8.2 per cent before treatment and who may have been at the beginning of a spontaneous remission. In both patients the initial marrow had shown considerable erythroid hyperplasia before treatment.

There was no apparent proportionality in our material between the extent of the increase and the maximal erythroid hyperplasia achieved on the one hand and the reticulocyte response and hemoglobin response on the other. This lack of correlation may be ascribed to a number of factors operating in our patients; namely, the effects of transfusions, the presence of infection and the effectiveness of the treatment directed against infection, the age differences, the factor of prematurity in one instance (Case 7), and the possible effects of coexisting deficiencies.

#### SUMMARY AND CONCLUSIONS

Serial studies of the bone marrow in fifteen patients with infantile megaloblastic anemia responding specifically to folic acid therapy as shown by reticulocytosis and rise in hemoglobin and red cell counts demonstrated a spectrum of gradual and progressive aberrations in the appearance of the erythroblasts. The patterns ranged from predominantly normoblastic to frankly megaloblastic cells with many intermediate types which could not be classified in either category. The classic megaloblastic dysplasia is interpreted as only an extreme deviation from normal structure; earlier, less striking changes are significant as shown by the therapeutic test. By the use of more liberal criteria regarding the classification of erythroblasts and by the recognition of constant and specific changes in the granulocytes of the marrow, the disturbance resulting from a marrow deficiency in hemopoietic principle can be detected before the classic megaloblast stage has developed. The lack of hematopoietic principle leads to a disturbance in mitosis, and specific therapy results in a prompt return to normal mitotic activity.

Within twenty-four hours after the intramuscular injection of folic acid clear-cut morphologic changes take place in the marrow of these patients and a response in the peripheral blood can be predicted. Folic acid produces a prompt return to the normoblastic type of erythropoiesis. The immediate transformation of all but the most nearly mature megaloblasts suggests that the megaloblastic dysplasia of the individual cells is reversible so long as the

cell remains capable of mitosis. In the course of the transformation the pathologic cells pass again through an intermediate stage.

In megaloblastic anemia of infancy the total myeloid-erythroid volume of aspirated marrow samples shows no correlation with the degree of anemia and is not a reliable index of the response to therapy, but the calculated volume of erythroid cells shows a direct proportionality to the severity of the anemia and a significant rise in response to therapy.

#### REFERENCES

1. Zuelzer, W., and Ogden, F.: Folic Acid Therapy in Macrocytic Anemias of Infancy, *Proc. Soc. Exper. Biol. & Med.* 61: 176, 1946.
2. Zuelzer, W.: Folic Acid Therapy in the Anemias of Infancy and Childhood, *J. A. M. A.* 131: 7, 1946.
3. Zuelzer, W., and Ogden, F.: Megaloblastic Anemia in Infancy, *Am. J. Dis. Child.* 71: 211, 1946.
4. Downey, H.: For reference, see Wintrobe, M. M.: *Clinical Hematology*, Philadelphia, 1944, Lea & Febiger.
5. Jones, O. P.: Cytology of Pathologic Marrow Cells With Special Reference to Bone Marrow Biopsies. In Downey, H.: *Handbook of Hematology*, New York, 1938, Paul B. Hoeber, Inc.
6. Osgood, E., and Seaman, A.: The Cellular Composition of Normal Bone Marrow as Obtained by Sternal Puncture, *Physiol. Rev.* 24: 46, 1944.
7. Rohr, K.: *Das menschliche Knochenmark*, Leipzig, 1940, Georg Thieme.

# FOLIC ACID THERAPY IN NONTROPICAL SPRUE: RESULTS OF TREATMENT IN SEVEN CASES

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DURING the latter part of 1945 Spies and associates<sup>1</sup> reported that synthetic folic acid exerted a favorable influence on the course of macrocytic anemias. Included in their study were patients with tropical sprue in whom the favorable response was not only hematologic but also general. The symptoms referable to the alimentary tract in these patients also tended to subside when folic acid was employed. Subsequent reports include those of Darby and of Spies and their associates<sup>1-4</sup> and occasional instances of sprue in papers of other workers who were chiefly concerned with pernicious anemia.

Davidson, Girdwood, and Innes<sup>5</sup> recently reported results of short periods of treatment with folic acid in ten patients with tropical sprue, idiopathic steatorrhea, and celiac disease. They were primarily interested in the hematologic response which they found disappointing. The clinical response was excellent in the patients with tropical sprue and idiopathic steatorrhea, but improvement was lacking in the patients with celiac disease. Studies of fat balance were made before and after treatment in seven patients (including one with celiac disease). In only one patient was there improvement in absorption of fat.

Nontropical sprue is a condition characterized by diarrhea, steatorrhea, and other gastrointestinal symptoms, macrocytic anemia, glossitis, nutritional manifestations including loss of weight, weakness, hypocalcemia, osteoporosis, tetany, hypoproteinemia, edema, and other changes. These manifestations may vary considerably from patient to patient. The disease, furthermore, is characterized by a chronic progressive course and often prolonged remissions. Treatment in the past has been based chiefly on the use of a low fat diet and administration of liver extract, calcium, and the various vitamins. In general, the results of treatment have been disappointing. In most cases remission occurred, but the tendency to spontaneous remission has rendered the interpretation of the value of therapeutic agents most difficult.

Stimulated by earlier reports of Spies and co-workers<sup>6</sup> our group has treated seven patients who had nontropical sprue with synthetic folic acid.\* These form the basis for this report. The criteria of nontropical sprue were fulfilled in the opinion of the several consultants who saw the patients. None of the patients had lived in a tropical country.

## PLAN OF STUDY

Control data were obtained before institution of treatment. The history included notes about the frequency, duration, and severity of the exacerbations

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\*The folic acid was kindly supplied by the Lederle Laboratories, Inc., Pearl River, N. Y.

and about the severity of the disease between exacerbations, including frequency and character of stools. Hematologic studies included sternal aspiration and examination, determination of the concentration of hemoglobin in grams, erythrocyte, leucocyte, and reticulocyte counts, and measurement of the erythrocytes with an erythrocytometer. The concentrations of calcium, phosphorus, total protein, albumin, and globulin in the serum, as well as values for phosphatase and prothrombin times, were determined. Roentgenograms of the skull, hands, and teeth for osteoporosis and of the small bowel to exclude ileitis and to check for the so-called deficiency pattern were secured. Roentgenologic examination of the small bowel was not repeated because changes during periods of improvement had not been observed in past experience. In four subjects the fecal solids, fat, and nitrogen were determined each day during the three-day test period while the patient was receiving a standard test diet of 101.6 Gm. of fat, 117.5 Gm. of protein, 269.6 Gm. of carbohydrate, and 2,463 calories. On completion of the preliminary survey a diet was used containing 50 to 100 Gm. of fat and 120 Gm. or more of protein and carbohydrates to raise the caloric intake to 3,000 to 3,500. Vitamins, including sufficient vitamin K to control hypoprothrombinemia, were given. The dosage of folic acid usually was 50 mg. daily by the intramuscular route. One patient received 180 mg. daily intramuscularly for two weeks while another received only 15 mg. orally from the start. Hematologic and chemical determinations on the blood were made each week; the reticulocyte count was taken every other day.

Five of the seven patients were kept under observation at the clinic for four weeks or longer. On dismissal from the clinic the patients continued to take folic acid intramuscularly or orally, the usual vitamins, and, in most cases, calcium lactate in doses of 1 dram (3.9 Gm.) three times daily. Some also subsequently were given liver extract. All were urged to use a high carbohydrate, high protein, low fat diet. The dosage of folic acid is indicated in the individual case reports and in Table I.

Some of the patients subsequently returned for observation and hematologic and chemical studies on the blood. In two subjects the losses of fecal solids, fat, and nitrogen were determined after eight and twelve months of treatment with folic acid. In two others the fecal components could not be determined because of refusal of the patient or because of his condition.

Each subject on his return home was requested to keep a diary in which was recorded the number of stools passed daily as well as other manifestations of the disease.

CASE 1.—A 51-year-old man complained of diarrhea of forty months' duration. The stools were steatorrheal in type and averaged about three daily except during exacerbations, when they numbered as many as ten and fifteen daily. He had lost much strength and 25 pounds (11.3 kilograms). No tetany had been experienced.

Examination revealed undernutrition, for the patient weighed only 110 pounds (50 kilograms), and pigmentation of the skin. Free hydrochloric acid (20 units, by Töpfer's method) was found on gastric analysis. Roentgenologic studies revealed a so-called deficiency pattern in the small intestine. When the standard test diet of 101.6 Gm. of fat and 117.5 Gm. of protein was used, the average daily loss of fat and nitrogen was, respectively, 41 and 5.8 Grams. Stools averaged seven per day during the test period (Table II). An oral glucose



TABLE I. HEMATOLOGIC DATA IN NINE CASES

CASE	DURATION OF TREATMENT	FOLIC ACID (MG.)	HEMOGLOBIN (GM./100 C.C.)	ERYTHROCYTES (MIL/CMM.)	MORPHOLOGY OF ERYTHROCYTES	THROMBOCYTOMETRIC ( $\mu$ )	LEUCOCYTES (% THROMBOCYTES)	SPLEENAL MARKING
1	Before		12.3	1 30	Macrocytosis		1	Normoblastic
	1 mo.	50	11.6	3 37	Macrocytosis	8.1 5.3	3.3	
	3 mo.	50	11.2	4.00	Macrocytosis	8.0 8.1		Normoblastic
	9 mo.	50	9.6	3 58	Macrocytosis	8.0		Normoblastic
2	Before		7.6	3 61	Normocytic hypochromic erythrocytes	7.1	1.7	Hyperplastic normoblastic
	1 mo.	50	8.25	3 61		7.6	3.0	
	9 mo.	15	6.4	1 01	Normocytic hypochromic erythrocytes	7.1		
3	Before		12.7	4 30	Macrocytosis	7.5 5.0	1.1 2.2	Normoblastic
	6 wk.	15 180	13.8	1 12	Macrocytosis	7.9 8.1	2.4	Megaloblastic
	Before		10.8	3.09	Macrocytosis		0.1 1.6	Normoblastic
	6 wk.	50	12.0	3.86	Macrocytosis	8.5 7.5	1.9	Macronormoblastic
5	Before		11.9	3 03	Macrocytosis	8.3		
	2 mo.	50	10.8	3.08	Macrocytosis	8.2	3.0	
	5 mo.	50	10.8	4.08	Macrocytosis	8.2		
	7 mo.	30	9.4	3.30	Macrocytosis	8.2	1.9	
6	Before		10.5	4.25	Macrocytosis	8.6		Normoblastic
	2 mo.	50	10.5	4.29	Macrocytosis	8.3		
	Before		11.0	5.26	Macrocytosis			Normoblastic
	12 mo.	15	9.8	4 60	Borderline macrocytosis		1.8	

TABLE II. FECAL SOLIDS, TOTAL LIPOIDS, AND TOTAL NITROGEN IN TWO CASES OF SPURVE BEFORE AND DURING THERAPY WITH FOLIC ACID

CASE	RELATION TO FOLIC ACID THERAPY		STOOLS: DAILY AVERAGE NUMBER DURING STUDY	AVERAGE DAILY VALUES*			
	BEFORE	DURING		SOLIDS (GM.)	TOTAL LIPOIDS (GM.)	TOTAL LIPOIDS (% SOLIDS)	TOTAL NITROGEN (GM.)
1	April, 1946		7†	141.7	41.0	28.9	5.8
		Jan., 1947	2	74.2	36.2	48.7	3.8
2	April, 1946		1	50.6	29.6	58.5	2.5
		Jan., 1947	1	81.2	43.9	54.1	2.6
Normal persons	Average		-	27.6	4.1	14.5	1.7
	Range			13.6-39.1	1.8-6.7	9.3-19.6	0.8-2.5

\*A standard test diet containing 101.6 Gm. of fat, 117.5 Gm. of protein, 269.6 Gm. of carbohydrate, and 2,163 calories. Marker technique employed.

†Study conducted during exacerbation of disease.

tolerance test presented a flat curve. The value for serum protein was 6.1 Gm. per 100 c.c.; and the albumin globulin ratio was 1.1:1. Serum calcium measured 8.8 mg. per 100 c.c.; there were 12.3 Gm. of hemoglobin per 100 c.c. of blood, and erythrocytes numbered 1,300,000 per cubic millimeter of blood, of which 1 per cent was reticulocytes. General macrocytosis was present. The sternal bone marrow was normoblastic. Folic acid was given intramuscularly in 50 mg. doses each day. During thirty days of observation the patient did not gain weight, strength, or sense of well being in spite of a daily intake of 3,000 to 3,500 calories. Frequency of stools did not change; the number varied from one to five daily. At the end of this period the value for hemoglobin was 11.6 Gm. per 100 c.c., and erythrocytes numbered 3,370,000 per cubic millimeter. The highest reticulocyte count was 3.3 per cent encountered on the sixth day of therapy. Throughout this period the erythrocytes measured 8.1 to 8.3  $\mu$  in diameter. Serum proteins then measured 6.8 Gm. per 100 c.c., the albumin globulin ratio was 1.09:1, and the value for serum calcium was 9.3 mg. per 100 cubic centimeters.

After dismissal the patient continued the nutritious diet and daily intramuscular injections of 50 mg. of folic acid. In spite of this treatment he returned in one month because of an acute exacerbation of the disease characterized by abdominal pain, distention, diarrhea, vomiting, abdominal tenderness, and loss of 10 pounds (4.5 kilograms). On examination he was acutely ill and gave evidence of dehydration, acidosis, and azotemia. With correction of these conditions the acute symptoms subsided. He remained under observation another month, during which time administration of the folic acid was continued. He gained a few pounds but mild diarrhea persisted. At the end of this period the concentration of hemoglobin was 11.2 Gm., erythrocytes numbered 4,000,000, macrocytosis persisted with erythrocytometer readings of 8 and 8.4  $\mu$ , and the value for serum proteins was 6.1 Gm. per 100 cubic centimeters. On dismissal he was instructed to take 50 mg. of folic acid daily by mouth. This treatment was continued for six more months, during which time the patient did not improve; mild diarrhea with exacerbations periodically continued, and gain in weight did not occur.

The patient then returned for further observation. At this time the value for hemoglobin was 9.6 Gm. and erythrocytes numbered 3,580,000. The general macrocytosis persisted. The erythrocytometer reading was 8.0  $\mu$ , the concentration of serum protein was 5.5 Gm. per 100 c.c., the albumin-globulin ratio was 1.1:1, and the value for serum calcium was 8.2 mg. per 100 cubic centimeters. Average daily loss of fecal solids, fat, and nitrogen again was determined (Table II); stools numbered two daily during the test period. Comparison of these data with similar data obtained before treatment disclosed that loss of fat was essentially the same but that nitrogen loss was less during the period after treatment. However, the improvement, in our opinion, is due not to the folic acid but to the state of the disease at the time the data were obtained. More fat was lost during the first period of study and during an exacerbation of the disease than during the second period of study which was made at a time when an exacerbation was not present. The differences are small and probably not significant.

*Comment.*—The course of the disease has not been changed by treatment with synthetic folic acid, diet, and vitamins. In fact the course has been chronic and gradually downhill in spite of treatment for nine months. Severe exacerbations have occurred; weight has not been gained. The degree of anemia and of hypocalcemia has fluctuated slightly but has remained essentially the same at the last examination as at the first one. Hypoproteinemia has appeared.

CASE 2—The patient, a woman first seen when she was 29 years of age, had had a lifelong tendency to loose stools but this had been worse during the four years preceding admission. Weight had declined from 130 to 103 pounds (59.0 to 46.7 kilograms), slight edema had occurred, and tetany had been noted for one year. Stools were 64 to 74 per cent fat (dry weight). Gastric analysis revealed 20 units of free acid. Roentgenologic examination of the small bowel revealed a deficiency pattern. The concentration of hemoglobin was 15 Gm per 100 c.c. of blood; erythrocytes numbered 4,190,000; the value for serum protein

was 5.6 Gm., and for serum calcium, 6.6 mg. per 100 cubic centimeters. Until the next period of observation (period of the present study) when the patient was 33 years old, mild diarrhea had continued; an average of three to four loose stools were passed daily. Slight edema and latent tetany also continued. Weight was 101 pounds (45.8 kilograms). Laboratory studies revealed general osteomalacia of the bones, a flat oral glucose tolerance curve, 7.1 mg. of calcium and 5.9 Gm. of proteins per 100 c.c. of serum, albumin-globulin ratio of 2.2:1, 7.6 Gm. hemoglobin, 3,610,000 erythrocytes, hypochromic normocytic erythrocytes, an erythrocytometer reading of 7.4  $\mu$ , and hyperplastic normoblastic sternal marrow. For four weeks a diet of 3,000 to 3,500 calories (fat, 75 Gm.; protein, 150 Gm.) was taken well without significant diarrhea. During this time 50 mg. of folic acid were given intramuscularly daily. A gain of five pounds (2.3 kilograms) occurred. This gain in weight was credited to increased intake of food and to rest. At the end of this period the value for hemoglobin was 8.25 Gm., erythrocytes numbered 3,610,000, the erythrocytometer reading was 7.6  $\mu$ , the value for serum protein was 6.6 Gm., the albumin-globulin ratio was 2.18:1 and serum calcium, 7.9 mg. per 100 cubic centimeters. After the institution of folic acid therapy the highest reticuloocyte count was 3 per cent on the fifth day of treatment. In general, the patient noted no subjective improvement. She returned home with instructions to continue the highly nutritious diet (3,500 calories with 75 Gm. of fat and 150 Gm. of protein) and to take a multivitamin tablet, iron, and 15 mg. of folic acid daily. At first the folic acid was taken intramuscularly and later, orally.

When the patient returned eight months later, she stated that her weight had increased to 113 pounds (51.3 kilograms) and then had dropped to 96 (43.5 kilograms) because of inability to eat during an exacerbation three months previously. Diarrhea had been troublesome only during the exacerbation, but tetany occurred at intervals and variable edema had been present. Folic acid therapy had been interrupted only for a time during relapse. The weight was 96 pounds. Laboratory studies revealed 6.4 Gm. of hemoglobin per 100 c.c. of blood, 4,010,000 erythrocytes, hypochromic normocytic erythrocytes, an erythrocytometer reading of 7.4  $\mu$ , 5.3 Gm. of calcium and 5.3 Gm. of protein per 100 c.c. of serum, and an albumin-globulin ratio of 2.32:1. The patient was placed on the standard test diet both before and after treatment with folic acid, and the values for fecal fat and nitrogen were determined. The severity of the disease apparently was the same during each period of study, the stools numbering one or two daily. After treatment the average daily loss of fats was greater than before treatment but the loss of nitrogen was not significantly different. Therapy with folic acid did not decrease the loss of fat and nitrogen in the feces.

*Comment.*—Slight gain in weight and improvement in the degree of hypoproteinemia and hypocalcemia occurred, as is so often the case in nontropical sprue, with or without treatment. The temptation to ascribe the improvement to folic acid is disputed by the occurrence of a severe exacerbation while receiving 15 mg. of the drug intramuscularly or orally each day. The course of the disease has not been altered.

**CASE 3.**—A 37-year-old woman had had symptoms of nontropical sprue for three years. Diabetes mellitus had been discovered about the same time. One and one-half years before admission an exacerbation of the sprue syndrome had occurred, during which glossitis was marked and weight had declined from 105 to 78 pounds (47.6 to 35.4 kilograms). Three months later tetany was noted. At the time of admission the patient was not suffering from diarrhea but the stools appeared fatty. Weight was 81 pounds (36.7 kilograms). Laboratory findings were as follows: glycosuria, 1.39 per cent; blood sugar, 185 to 226 mg. per 100 c.c.; osteoporosis of the skull, spinal column, and bones of the hands; and 14 units of free gastric acid. There was roentgenologic evidence of a deficiency pattern in the small intestine. On the standard test diet the average daily losses of fecal solids, fat, and nitrogen were, respectively, 67, 25, and 3 grams. The value for serum calcium was 7.9 mg. and for serum protein, 6.2 Gm. per 100 c.c.; the albumin-globulin ratio was 1.46:1; the concentration of hemoglobin was 12.7 Gm. per 100 c.c.; erythrocytes numbered 4,300,000, of which 1.1 to 2.2

per cent were reticulocytes; general macrocytosis was indicated by erythrocytometer readings of 7.8 to 8.0 microns. A diet containing 2,700 to 3,000 calories, with fat limited to 100 Gm. or less daily, was taken well. The requirements for insulin were met. A gain in weight from 81 to 103 pounds (36.7 to 46.7 kilograms) occurred in seven weeks; about 10 pounds (4.5 kilograms) were gained during the week of observation before folic acid was started. Usually the patient had two or three stools daily but at times constipation was present. After one week's observation folic acid was administered intramuscularly in doses of 15 mg. daily for two weeks, then 30 mg. twice daily for one week, 90 mg. twice daily for two weeks, and finally 90 mg. daily for one week. During this time the gain in weight just mentioned continued. At completion of observation the value for hemoglobin was 13.8 Gm. and erythrocytes numbered 4,120,000; macrocytosis had persisted for the erythrocytometer reading varied from 7.9 to 8.1 microns. The highest reticulocyte count was 2.4 per cent on the seventh day of treatment. Sternal marrow was normoblastic at the time of completion of observation. The value for serum protein was 7.1 Gm., the albumin-globulin ratio was 1.6:1, and the value for serum calcium was 7.9 mg. per 100 cubic centimeters. After return home the patient continued to take folic acid intramuscularly in 15 mg. doses daily and progressed well for three months, when an acute exacerbation of the sprue syndrome developed which led to death.

*Comment.*—Synthetic folic acid did not alter the course of the disease in this case.

CASE 4.—The patient, a woman, was first seen when she was 24 years of age. Symptoms of nontropical sprue had been present for seven months. Diarrhea, loss of 20 pounds (9.1 kilograms), glossitis, transitory edema, and tetany had occurred. The concentration of hemoglobin was 43 per cent (Dare); erythrocytes numbered 4,060,000. The value for serum protein was 5.45 Gm. and for calcium, 8.7 mg. per 100 cubic centimeters. The value for free gastric acidity was 30 units (Töpfer's method). Stools contained an excess of fat.

When the patient returned seven years later at the age of 31, weakness and tetany were the chief complaints. Diarrhea was present. The blood presented a picture of marked hypochromic macrocytic anemia, the concentration of hemoglobin was 7.0 Gm. per 100 c.c. of blood, and erythrocytes numbered 3,180,000 per cubic millimeter. Mild symptoms continued and at the time of the next visit at the age of 41 the patient complained that diarrhea was troublesome. Weight was then 103 pounds (46.7 kilograms). Free gastric acids measured 36 units. Roentgenologic examination of the small intestine revealed a deficiency pattern. On the standard test diet, the average daily loss of fecal solids, fat, and nitrogen were, respectively, 38, 14, and 2 grams. An oral glucose tolerance test gave a flat curve. The value for serum calcium was 8.0 mg. and for serum protein, 6.2 Gm. per 100 c.c.; the albumin-globulin ratio was 2.06:1. The concentration of hemoglobin was 10.8 Gm. per 100 c.c. and erythrocytes numbered 3,090,000, of which 0.4 to 1.6 per cent were reticulocytes. Macrocytosis was marked. Sternal aspiration revealed megaloblastic marrow. After intramuscular administration of 50 mg. of folic acid daily slight reticulocytosis occurred, the highest reading being 4.9 per cent on the fifth day of treatment. Two weeks later the sternal marrow was normoblastic. After six weeks the value for hemoglobin was 12.0 Gm. per 100 c.c. and erythrocytes numbered 3,860,000. Erythrocytometer readings during the period of treatment varied from 8.5 to 7.8 microns. At the end of the period of observation (six weeks) the value for serum calcium was 9.1 mg. and for serum protein, 7.7 Gm. per 100 c.c.; the albumin-globulin ratio was 1.34:1. During this period of treatment the patient took a 3,500 calorie diet daily (100 Gm. of fat and 120 Gm. of protein). Several mild exacerbations of the diarrhea occurred (seven to ten stools daily); weight increased to 106 pounds (48.1 kilograms), a gain of only 3 pounds (1.4 kilograms).

Following dismissal the patient continued to use a high caloric diet (fat, 50 Gm. and protein, 160 Gm.) and to take 15 mg. of folic acid orally each day. The exacerbations of diarrhea, as well as varying degrees of edema and tetany, have continued to occur, and, on one occasion, a peculiar generalized paralysis occurred. On several occasions during this eight-

month interval hospitalization for treatment became necessary. Liver extract, calcium, vitamins, and intravenously administered amino acids have been used together with folic acid.

*Comment.*—The condition of this patient remains pitiful in spite of treatment not only with folic acid, but with other agents as well.

**CASE 5.**—The patient, a 42-year old woman, had been anemic at varying times for twenty years and had always had a tendency to loose stools. For ten years, the stools had at times been more frequent than before and were described as foamy. The chief complaint was fatigue and weakness, and she had lost 5 pounds (3.6 kilograms) in three months. Weight was 128 pounds (58.1 kilograms). Laboratory studies revealed 11.9 Gm. of hemoglobin, 3,030,000 erythrocytes, general macrocytosis with an erythrocytometer reading of 8.3  $\mu$ , 9.1 mg. of serum calcium and 6.2 Gm. of serum protein per 100 c.c., and an albumin globulin ratio of 19.1. There was a deficiency pattern in the small intestine on roentgenologic examination. Gastric analysis revealed 52 units of free acid; the oral glucose tolerance curve was flat and the stools contained 31 per cent fat (dry weight) while the patient was taking only 100 Gm. of fat each day. Sternal marrow was macronormoblastic in type. Folic acid in doses of 50 mg. was given orally each day. The highest reticulocyte count was 3 per cent on the eighth day of treatment. After three weeks of treatment the value for hemoglobin was 10.2 Gm. and erythrocytes numbered 3,840,000. After two months of treatment macrocytosis was still present, and the erythrocytometer reading was 8.2 microns. The patient's strength was good but no weight had been gained and episodes of diarrhea continued. Five months after institution of treatment, during an episode of diarrhea, a hemorrhagic state of extensive degree developed and was promptly controlled by administration of vitamin K. During this episode the patient was hospitalized elsewhere and a loss of 10 pounds (4.5 kilograms) in weight occurred. On her return to the clinic the value for hemoglobin was 10.8 Gm. and erythrocytes numbered 4,080,000. Her condition remained fairly stationary for the next month and then the diarrhea became much worse, six to eight stools occurring daily. Weight dropped to 105 pounds (47.6 kilograms). She was again hospitalized and additional measures, including liver extract, were instituted to control symptoms. Folic acid was given intramuscularly instead of orally. Improvement gradually developed. The value for hemoglobin was 9.4 Gm., erythrocytes numbered 3,300,000, the erythrocytometer reading was 8.2  $\mu$ , and 1.9 per cent of the erythrocytes were reticulocytes. Stools contained 32.7 per cent fat by dry weight.

*Comment.*—In addition to absence of response hematologically or clinically to folic acid this patient presented an episode of hypoprothrombinemia with hemorrhage and an acute exacerbation of the intestinal symptoms while faithfully adhering to a regimen which included folic acid.

**CASE 6.**—A 53 year old woman had been seen on numerous occasions during the preceding ten years. At the first observation in 1936 cholecystectomy was performed for chronic cholecystitis with cholelithiasis. Exploration of the abdomen was otherwise normal. Moderate gastric retention was present preoperatively and at intervals postoperatively. During convalescence diabetes mellitus became manifest but was readily controlled. Weakness was also a prominent complaint. When the patient was 51 years of age, abdominal cramps, distention, and spells of diarrhea developed. Stools were bulky and light in color. Weight had declined from 99 to 88 pounds (44.9 to 39.9 kilograms). Blood counts which had previously been normal now revealed anemia. There were 11.6 Gm. of hemoglobin per 100 c.c. of blood, 3,340,000 erythrocytes, 6.3 mg. of serum calcium and 6.8 Gm. of serum protein per 100 c.c., and the albumin globulin ratio was 18.1. Stools contained 48.6 per cent fat. The patient received the usual treatment for sprue consisting of a low fat diet, liver extract, calcium lactate, and vitamin supplements. When seen a year later (the time of the present study) symptoms had persisted and weight had declined to 81 pounds (36.7 kilograms). No edema or tetany had been noted and the diabetes had not been a problem. Laboratory study now disclosed 10.5 Gm. of hemoglobin per 100 c.c., 4,250,000 erythrocytes, and general macrocytosis with an erythrocytometer reading of 8.6 microns. The value for serum protein was

6.1 Gm. per 100 c.c.; the albumin globulin ratio was 1.7:1; the value for serum calcium was 8.2 mg. per 100 cubic centimeters. The fecal solids were 65.3 per cent fat. Sternal marrow appeared normoblastic. In addition to previous therapy, 50 mg. of folic acid were given daily by mouth. Two months later the symptoms and the general status of health and bowel function had not changed. The value for hemoglobin was 10.5 Gm. per 100 c.c., and erythrocytes numbered 1,290,000. The general macrocytosis had persisted; the erythrocytometer reading was 8.3 microns.

*Comment.*—Folic acid had evidently accomplished little in this period.

CASE 7.—A 17-year old girl had had episodes of diarrhea since infancy. These occurred about once a month and lasted three to five days; the stools, which numbered ten to twenty daily, were greasy and foamy. Growth was much retarded. She was 4 feet, 10 inches (147.3 cm.) tall and weighed 79 pounds (35.8 kilograms); menses had not been established. Edema had been noted on several occasions. The occurrence of tetany was questionable. The value for hemoglobin was 11 Gm. per 100 c.c., erythrocytes numbered 5,260,000, and macrocytosis was present. The stools were 52.7 per cent fat (dry weight). The value for serum protein was 4.8 Gm. per 100 c.c.; the albumin globulin ratio was 1.4:1. The concentration of serum calcium was 8.5 mg. per 100 c.c. and the sternal marrow was normoblastic. A regimen consisting of 3,000 calories per day (50 Gm. of fat), liver extract (1 c.c. weekly), and folic acid (15 mg. daily by mouth) was instituted.

The patient returned twelve months later. Monthly exacerbations of diarrhea had continued until two months prior to this visit. Some of the exacerbations were the most severe she had had. She had gained 14 pounds (6.4 kilograms) and had grown 3½ inches (9 cm.). The menstrual periods had not become established although pelvic pain for one to three days each month had occurred. Pigmentation had lessened. The breasts were still undeveloped; some pubic hair was present. The concentration of hemoglobin was 9.8 Gm per 100 c.c. of blood; erythrocytes numbered 4,600,000 per cubic millimeter, of which 1.8 per cent were reticulocytes. Some hypochromasia and a borderline type of macrocytosis were noted in the blood smear. The value for protein in the serum was 5.9 Gm. per 100 c.c.; the albumin globulin ratio was 1.99:1. The concentration of serum calcium was 9.5 mg. per 100 cubic centimeters. The stage of bone development corresponded to that of a child 13 years old.

*Comment.*—Definite improvement occurred in this case and was attributed to improvement in nutrition. It is impossible to determine whether this was due to the improved intake of food or to a natural remission in the disease or to the results of medication. A further period of treatment will be required to determine the principal factor in the improvement, and such a conclusion may not be possible even then.

#### COMMENT

Most important in the discussion of these cases is a consideration of the general status of the patients' health. Only two patients have shown any significant gain in weight or improvement in strength or sense of well-being (Cases 3 and 7). One patient (Case 3) had had symptoms of nontropical sprue and diabetes mellitus for three years. No diarrhea was present on admission although the stools contained excess fat. Before treatment with folic acid was started she had gained 10 pounds and further gain was accomplished after this treatment was instituted. This gain was considered to be due largely to an increased intake of food and control of the diabetes as the sprue was in remission at the time of admission. At the time of dismissal the patient was taking 15 mg. of folic acid daily by mouth and she progressed well for three months.

Then an exacerbation of the sprue syndrome resulted in death. The other patient (Case 7) showed definite improvement but it is not possible to state the chief factor responsible for this. The patient in Case 1, who gave a three-year history of sprue, continued to take 50 mg. of folic acid daily intramuscularly at home for one month. At the end of this time an exacerbation of the disease caused him to return to our care. He was in a critical condition as evidenced by dehydration, acidosis, and azotemia. In spite of continuation of treatment with folic acid as well as other therapeutic management for six months, his general condition did not improve nor intestinal manifestations decrease. One patient (Case 4) who had had indications of sprue for seventeen years continued to have exacerbations with varying degrees of edema and tetany, and on one occasion a peculiar paralysis, while taking 15 mg. of folic acid daily by mouth. This patient has required frequent hospital admissions during the subsequent eight months. The patient in Case 5, while taking 50 mg. of folic acid daily for six months, has had an episode of hypoprothrombinemia (not uncommon in nontropical sprue) with hemorrhagic manifestations and a subsequent acute exacerbation of the intestinal symptoms of the disease. Two patients (Cases 2 and 6) did not improve during nine and two months, respectively.

In general, the diarrhea and steatorrhea present before treatment with folic acid have continued during treatment, often with severe flare-ups, and there has been no gain in weight, strength, or sense of well-being that could not be attributed to remissions in the disease and improved intake of food. The levels of calcium or protein in the blood and manifestations of tetany or edema, when present, have not been improved permanently in any case, even with improved intake of food or other procedures.

Some anemia was present in all cases at the time of admission, but the value for hemoglobin was 10 Gm. or more in all but one. The lowest erythrocyte count was 3,030,000. No significant changes in blood counts were obtained from treatment with folic acid. Details are given in Table I. General macrocytosis was present in all patients except one, and this patient (Case 2) had the most marked anemia. The macrocytosis persisted after treatment with folic acid in each of the five cases in which re-examination has been done. No change in the erythrocytometer reading was encountered except in Case 4. In this instance the size of the erythrocytes decreased somewhat coincident with a change in the sternal marrow from megaloblastic to normoblastic type. The highest reticulocyte count after commencement of treatment with folic acid was 4.9 per cent on the fifth day of treatment. In general there was little change in the reticulocyte count. However, the degree of anemia on admission was not such that a great response would be expected. In one case megaloblastic bone marrow present before treatment became normoblastic after treatment. In four other cases the sternal marrow was normoblastic before treatment was started. Some of these patients undoubtedly had taken liver extract at varying times before the observations at the clinic. In general, and in spite of this, the hematologic response in this group of cases has been disappointing; the anemia has not improved and the macrocytosis has persisted.



## SUMMARY

The following statements seem justified from our observation of this group of seven cases of nontropical sprue. Treatment with folic acid has not resulted in improvement in the sense of well-being or in gain in weight or strength that could not be accounted for by increased intake of food and natural remission of the disease. The frequency or severity of the exacerbations of the intestinal manifestations of the disease has not changed. The hematologic response has been disappointing since anemia and macrocytosis have persisted. All this is in contrast to the favorable results reported in the literature regarding response of tropical sprue to treatment with folic acid. Whether the two conditions are different or whether the condition in our cases had advanced to an irreversible stage and become resistant to treatment with folic acid is impossible to state. These results indicate that, in some cases at least, nontropical sprue does not respond favorably to treatment with folic acid.

## REFERENCES

1. Spies, T. D., Vilter, C. F., Koch, Mary B., and Caldwell, Margaret H.: Observations of the Anti-Anemic Properties of Synthetic Folic Acid, *South. M. J.* 38: 707-709, 1945.
2. Darby, W. J., and Jones, E.: Treatment of Sprue With Synthetic L. Casei Factor (Folic Acid, Vitamin M), *Proc. Soc. Exper. Biol. & Med.* 60: 259-260, 1945.
3. Darby, W. J., Jones, Edgar, and Johnson, H. C.: The Use of Synthetic L. Casei Factor in the Treatment of Sprue, *Science* 103: 108, 1946.
4. Spies, T. D., Lopez, G. G., Menendez, J. A., Minnich, Virginia, and Koch, Mary B.: The Effect of Folic Acid on Sprue, *South. M. J.* 39: 30-32, 1946.
5. Davidson, L. S. P., Girdwood, R. H., and Innes, Elizabeth M.: Folic Acid in the Treatment of the Sprue Syndrome, *Lancet* 1: 511-515, 1947.
6. Spies, T. D., Milanes, Fernando, Menéndez, Aristides, Koch, Mary B., and Minnich, Virginia: Observations on the Treatment of Tropical Sprue With Folic Acid, *J. Lab. & Clin. Med.* 31: 227-241, 1946.

## URINARY PHENOLS IN PERNICIOUS ANEMIA

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**A**LTHOUGH physiologic changes in pernicious anemia are widespread, affecting the hematopoietic, digestive, and nervous systems, the development and course of the disease have not been shown to be closely associated with any specific biochemical disturbance. Therefore, evidence suggesting that the metabolism of the aromatic amino acid, tyrosine, may be altered in pernicious anemia is of considerable interest.

Observations bearing on the metabolic behavior of tyrosine in pernicious anemia indicate that in this disease blood phenol levels are high,<sup>1</sup> the urinary levels of the volatile phenols are lower in relapse than in remission,<sup>2</sup> and the urinary excretion of total phenolic compounds is increased in untreated cases.<sup>3</sup> Jacobson and SubbaRow<sup>4</sup> have reported that 1-tyrosine increases the therapeutic activity of their primary antipernicious anemia factor. Since tyrosine is the presumed precursor of melanin pigment, the pigmentation changes frequently observed in pernicious anemia, which include graying of the hair and vitiligo, might also be considered as supportive evidence of the altered metabolism of tyrosine in this disease.

The present study was made to investigate further the metabolism of tyrosine in pernicious anemia by a fractionation of urinary phenolic compounds before and during initial treatment of the disease.

### CLINICAL MATERIAL AND METHODS

Seven patients with pernicious anemia were employed in this study. They had not received antianemia therapy for at least six months prior to the present study. All of them had macrocytic anemia with erythrocyte levels below 3,000,000 per cubic millimeter. Histamine refractory achlorhydria was demonstrated in each instance, and all of the patients responded in the expected manner to the administration of antipernicious anemia medication.

Urine specimens were collected for two twenty-four hour periods immediately preceding the institution of therapy and for several twenty-four hour periods at intervals thereafter.

The urinary phenolic compounds were fractionated according to an unpublished method of Shaeter and Lewis.<sup>5</sup> By this method, after preliminary hydrolysis, the urine was extracted with ether and the ether-soluble portion extracted first with sodium bicarbonate and then with sodium hydroxide. Phenols were determined by the Medes<sup>6b</sup> modification of the method of Folin and Ciocalteu<sup>6a</sup> on each of the three fractions resulting from this procedure: (1) the ether-insoluble fraction, (2) the ether-soluble-bicarbonate-soluble fraction, and (3) the ether-soluble-bicarbonate-insoluble-hydroxide-soluble fraction. This last fraction will be referred to as the ether-soluble-hydroxide-soluble fraction.

The ether-insoluble fraction contains any tyrosine excreted as such in the urine. The ether-soluble urinary phenolic compounds which are extracted with the weak alkali, sodium

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bicarbonate, are assumed to be, for the most part, aromatic hydroxy acids. Phenol and cresol are the only known phenols present in the urine in considerable amounts which are so weakly acidic that they cannot be extracted from another solution except with a strong alkali such as sodium hydroxide.

## RESULTS

In Table I the results of the fractionation of urinary phenolic compounds before and after the institution of specific therapy are presented for seven patients with pernicious anemia.

TABLE I. FRACTIONATION OF URINARY PHENOLS IN PERNICIOUS ANEMIA; EFFECT OF THERAPY

PATIENT	DATE	ETHER-SOLUBLE PHENOLS		RATIO A:B	ETHER- INSOLUBLE PHENOLS C (MG.)	TOTAL PHENOLS A + B + C (MG.)
		NaHCO <sub>3</sub> FRACT. A (MG.)	NaOH FRACT. B (MG.)			
G. O.	8/1	318	81	3.8	63	465
	8/2	351	105	3.2	48	504
	8/3*					
J. O.	8/4	82	62	1.3	32	176
	8/6	58	154	0.1	10	252
	5/18	402	25	16.1	48	475
	5/19	362	50	7.2	50	462
	5/20*					
	5/21	70	63	1.1	48	181
	5/24	34	64	0.5	27	125
S. T.	5/27	58	76	0.8	32	166
	6/7	264	32	8.2	24	320
	6/8	283	70	4.0	45	398
	6/9*					
	6/11	91	54	1.9	20	165
	6/14	56	41	1.3	23	120
	1/7	107	41	2.6	35	183
W. A.	1/8	106	52	2.0	31	189
	1/10*					
	1/11	64	60	1.1	40	164
S. N.	1/15	40	104	0.4	33	177
	1/8	240	41	6.0	45	326
	1/9	231	47	5.0	51	329
	1/10*					
	1/12	58	51	1.1	34	143
B. I.	1/14	56	48	1.2	39	143
	1/19	64	61	1.0	32	157
	2/28	245	79	3.2	48	372
	2/29	294	81	3.6	56	431
	3/1*					
	3/4	81	84	1.0	41	206
	3/8	57	101	0.6	48	206

\*Treatment instituted on this day. In all patients except B. I. this treatment was 1 c.c. of 15 unit liver extract by intramuscular injection three times weekly. In the case of B. I. the treatment was 40 Gm. of ventriculin daily.

Values expressed as milligrams of tyrosine excreted per twenty-four hours.

In every patient except one (W. A.) the excretion of total phenols was greater in the relapse than in the remission state. This lowering of phenolic excretion levels occurred whether remission was induced by a liver extract or by desiccated stomach (ventriculin). The rapidity with which the excretion of the phenols is decreased should be noted. The decrease occurs within forty-eight hours after the institution of therapy and precedes any change in the corpuscular elements of the blood, including reticulocytosis. These results are

in agreement with our preliminary observations on the excretion of keto acids and hydroxyphenyl compounds by four patients with pernicious anemia which are not included in the present study.<sup>3</sup>

The increased excretion of total phenols in the relapse period is wholly attributable to an increase in the excretion of ether-soluble-bicarbonate-soluble phenols, the fraction containing the hydroxyphenyl acids. The identity of this acid, or acids, has not been established. Although in a previous report,<sup>3</sup> using the method of Penrose and Quastel,<sup>4</sup> we obtained a greater keto acid excretion during the relapse period of pernicious anemia, in this series of patients we were unable to confirm this observation consistently and, in several cases, tests for keto acids in the ether-soluble urine fraction gave negative results.

The ether-soluble-hydroxide-insoluble fraction either remained the same or increased somewhat after therapy. When an increase occurred, it was more apparent in the urine samples collected several days after therapy was first administered. In this connection, Volterra<sup>2</sup> also observed in pernicious anemia an increase in volatile phenols when remission was induced.

An effect of antipernicious anemia therapy to which there is no exception in the cases presented here is the reduction in the ratio of the ether-soluble-bicarbonate-soluble fraction to the ether-soluble-hydroxide-soluble fraction. (This ratio is included in the results in Table I). This ratio reduction occurred even in patient W. A., in whom the total phenol level was not affected.

From Table I it can be seen that the ether-insoluble phenolic fraction remained at a relatively constant level before and during treatment.

The values for total phenols as well as for the various fractions determined during the remission phase of pernicious anemia show good agreement with the values obtained for normal subjects.<sup>7, Table I</sup> It is, therefore, apparent that in

TABLE II. EFFECT OF ASCORBIC ACID ON URINARY PHENOLIC EXCRETION IN UNTREATED PERNICIOUS ANEMIA

PATIENT	DAY	ASCORBIC ACID SUPPLE- MENT (MG.)	PLASMA ASCORBIC ACID (MG. %)	ETHER-SOLUBLE PHENOLS		RATIO A:B	ETHER- INSOLUBLE PHENOLS C (MG.)	TOTAL PHENOLS A + B + C (MG.)
				NaHCO <sub>3</sub> FRACT. A (MG.)	NaOH FRACT. B (MG.)			
J. O.*	1	0	0.1	147	33	4.4	48	190
	2	0		158	29	5.5	52	195
	3	500		203	32	6.3	47	282
	4	500		402	25	16.1	48	475
	5	500		362	50	7.2	50	462
S. N.*	1	0	0.7	241	57	4.2	46	344
	2	0		246	42	5.1	49	337
	3	500						
	4	500		240	41	6.0	45	326
B. I.*	5	500	0.8	231	47	5.0	51	329
	1	0		301	61	4.7	41	406
	2	0		281	60	4.7	52	393
	3	500		287	78	3.7	48	393
	4	500		245	79	3.3	48	372
	5	500		294	81	3.6	56	431

Values expressed as milligrams of tyrosine excreted per twenty-four hours.

\*These patients are also included in Table I. Except for B. I. ascorbic acid supplement was continued during the parenteral administration of liver extract.

pernicious anemia in relapse, the excretion of total phenolic compounds and hence of hydroxyphenyl acids is increased above normal levels.

In one patient with pernicious anemia (J. O.) we observed a low excretion of phenols prior to therapy. This individual had a fasting plasma ascorbic acid value of 0.1 mg. per 100 c.c., determined by the method of Mindlin and Butler.<sup>9</sup> Since ascorbic acid has been shown to have an effect on the oxidation of tyrosine,<sup>10, 11</sup> 500 mg. of ascorbic acid were given daily for a three-day period and the phenol excretion was measured. The results are shown in Table II. On the second day of ascorbic acid administration there was a definite increase in the hydroxyphenyl acid fraction. Ascorbic acid was also given to two patients with untreated pernicious anemia who had normal plasma ascorbic acid values. No changes in either total phenols excreted or in any fraction thereof were observed following the administration of ascorbic acid (Table II).

#### DISCUSSION

Effective treatment of patients with pernicious anemia with either liver or stomach preparations results in a decrease in excretion of the phenolic fraction containing hydroxyphenyl acids to a level approximating that obtained in normal subjects. This is evidence that substances concerned with erythrocyte maturation participate, directly or indirectly, in the oxidation of phenolic compounds and hence of the amino acid, tyrosine, beyond the aromatic acid stage.

Three of the known vitamins have been shown to affect tyrosine metabolism in various biologic systems: ascorbic acid,<sup>10, 11</sup> pyridoxine as pyridoxal phosphate,<sup>12</sup> and pteroylglutamic acid.<sup>13</sup> Since these vitamins are either absent or present in very small quantities in purified liver extracts,<sup>14</sup> it does not seem possible that the effect of liver extract on phenol excretion can be ascribed to its vitamin content. A more attractive hypothesis is that the antipernicious anemia principle in liver extract activates one or more of these vitamins in some metabolic reaction concerning tyrosine.

In our experiments, administration of ascorbic acid produced no effect on phenol excretion in cases of pernicious anemia where the plasma level of ascorbic acid was normal. In one patient with a low plasma ascorbic acid value and phenolic excretion not increased above normal levels, ascorbic acid administration was associated with an increased phenol excretion. This result is seemingly a contradiction of the well-defined action of ascorbic acid in scorbutic guinea pigs<sup>10</sup> and premature infants<sup>11</sup> in lowering the high excretion levels of hydroxyphenyl acid metabolites. However, indications that close metabolic relationships may exist between the liver principle and ascorbic acid are to be found in clinical reports of "anemia of scurvy"<sup>15</sup> and also in reports of vitamin C having a favorable effect on hematopoiesis in pernicious anemia.<sup>16</sup>

The results of Levine, Gordon, and Marples<sup>11</sup> show that in one of four premature infants, liver extract containing no ascorbic acid resulted in a "transient but definite decrease in hydroxyphenyl compounds." There is a possibility, in view of the experiments reported here, that in this instance the liver principle was the effective agent in reducing phenol excretion. That a deficiency of erythrocyte maturing factors occurs in premature infants is indicated by

reports that such infants often have incompletely developed hematopoietic systems associated with macrocytic anemia.<sup>17, 18</sup>

This study was completed prior to the investigations concerning pyridoxal phosphate and pteroylglutamic acid in relation to tyrosine oxidation; hence the possible effect of these vitamins on phenol excretion in pernicious anemia patients was not determined.

The evidence for aberrant tyrosine metabolism in pernicious anemia leads to the speculation that some phenolic derivative may directly participate in the erythrocyte developmental processes and that the liver principle may affect red cell maturation by virtue of its action in the tyrosine metabolic cycle to produce this derivative. If the formation of some phenolic derivative is essential in the treatment of macrocytic anemias resulting from a maturation defect, this might explain why liver extract is not effective in treating macrocytic anemia occurring in cases of cirrhosis of the liver<sup>19</sup> since tyrosine is not properly metabolized when liver function is impaired.<sup>20</sup>

#### SUMMARY

The treatment of pernicious anemia with liver or stomach preparations decreases the urinary excretion of total phenols by a reduction in the phenolic fraction containing the hydroxyphenyl acids. There is both a relative and an absolute increase in the fraction containing phenol and *p*-cresol. The ether-insoluble fraction remains constant.

The values for both total phenols and hydroxyphenyl acids obtained in pernicious anemia during relapse are greater than those obtained with normal subjects.

No change in phenol excretion levels in pernicious anemia in relapse was observed following administration of ascorbic acid to patients having normal plasma ascorbic acid values. In one patient with a low plasma ascorbic acid value, an increase in phenol excretion levels occurred concomitant with ascorbic acid supplementation.

#### REFERENCES

1. Becher, E., Litzner, S., and Täglic, W.: Der Phenolgehalt des Blutes unter normalen und pathologischen Verhältnissen, *Ztschr. f. klin. Med.* 104: 182, 1926.
2. Volterra, M.: L'élimination des phénols volatils par les urines dans l'anémie pernicieuse, *Schweiz. med. Wchnschr.* 69: 627, 1939.
3. Swendseid, M. E., Burton, I. F., and Bethell, F. H.: Excretion of Keto Acids and Hydroxyphenyl Compounds in Pernicious Anemia, *Proc. Soc. Exper. Biol. & Med.* 52: 202, 1943.
4. Jacobson, B. M., and Subbarow, Y.: Studies of the Principle in Liver Effective in Pernicious Anemia; the Therapeutic Activity of Its Multiple Factors, *J. Clin. Investigation* 16: 573, 1937.
5. Shacter, B., and Lewis, H. B.: Unpublished data.
- 6a. Folin, O., and Ciocoteu, V.: Tyrosine and Tryptophane Determinations in Proteins, *J. Biol. Chem.* 73: 627, 1927.
- 6b. Medes, G.: A New Error of Tyrosine Metabolism; Tyrosinosis, The Intermediary Metabolism of Tyrosine and Phenylalanine, *Biochem. J.* 26: 917, 1917.
7. Swendseid, M. E., Wandruff, B., and Bethell, F. H.: Urinary Phenols in Normal Subjects; Effect of a Liver Extract, *J. LAB. & CLIN. MED.* 32: 1248, 1947.
8. Penrose, L., and Quastel, J. H.: Metabolic Studies in Phenylketonuria, *Biochem. J.* 31: 266, 1937.
9. Mindlin, R. L., and Butler, A. M.: Determination of Ascorbic Acid in Plasma; Macro-method and Micromethod, *J. Biol. Chem.* 122: 673, 1938.

10. Sealock, R. R., and Silberstein, H. E.: The Excretion of Homogentisic Acid and Other Tyrosine Metabolites by the Vitamin C-Deficient Guinea Pig, *J. Biol. Chem.* 135: 251, 1940.
11. Levine, S. Z., Gordon, H. H., and Marples, E.: Defect in Metabolism of Tyrosine and Phenylalanine in Premature Infants; Spontaneous Occurrence and Eradication by Vitamin C, *J. Clin. Investigation* 20: 209, 1941.
12. Gunsalus, I. C., Bellamy, W. D., and Umbreit, W. W.: A Phosphorylated Derivative of Pyridoxal as the Coenzyme of Tyrosine Decarboxylase, *J. Biol. Chem.* 155: 685, 1944.
13. Rodney, G., Swendseid, M. E., and Swanson, A. L.: Tyrosine Oxidation by Livers From Rats With a Sulfasuxidine-Induced Pteroylglutamic Acid Deficiency, *J. Biol. Chem.* 167: 395, 1947.
14. Clark, G. W.: Vitamin Content of Liver Extracts for Parenteral Use; Comparison of Crude and Concentrated Preparations, *Am. J. M. Sc.* 209: 520, 1945.
15. Vilter, R., and Woolford, R. M.: The Anemia in Eight Adult Patients With Scurvy, *J. LAB. & CLIN. MED.* 30: 388, 1945.
16. Dyke, S. C., Della Vida, B. L., and Delikat, E.: Vitamin C Deficiency in "Irresponsive" Pernicious Anaemia, *Lancet* 2: 278, 1942.
17. Abt, A. F., and Nagel, B. R.: Prophylaxis of Anemia of Premature Infants; Use of Various Agents in Attempt to Prevent Development of So-called Physiologic Anemia of Premature Infants; Preliminary Study, *J. A. M. A.* 98: 2270, 1932.
18. Josephs, H. W.: Anaemia of Infancy and Early Childhood, *Medicine* 15: 307, 1936.
19. Wintrobe, M. M.: The Relation of Disease of Liver to Anemia; Type of Anemia, Response to Treatment, and Relation of Type of Anemia to Histopathologic Changes in Liver, Spleen and Bone Marrow, *Arch. Int. Med.* 57: 289, 1936.
20. Lichtman, S. S.: Origin and Significance of Tyrosinuria in Disease of the Liver, *Arch. Int. Med.* 53: 680, 1934.

# URINARY PHENOLS IN NORMAL SUBJECTS; EFFECT OF A LIVER EXTRACT

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THIS paper serves as a comparison study for the preceding communication<sup>1</sup> and reports the fractionation of the urinary phenols excreted by normal subjects and the effect thereon of a liver extract.

## METHODS AND MATERIAL

The urinary phenol values were determined according to previously described methods<sup>1</sup> on five normal subjects, medical students and dietitians, before and after the injection of liver extract. Throughout this period, to eliminate variations in phenol excretion due to dietary changes, these subjects were kept on a regulated diet similar to that received by the pernicious anemia patients discussed in the preceding report. This diet, on a 2,400 calorie basis, supplied approximately 85 Gm. of protein, 129 Gm. of fat, and 226 Gm. of carbohydrate. Extra calories were provided when needed to maintain weight by increasing the amount of fat and carbohydrate. The diet was supplemented with 200 mg. of ascorbic acid.

The liver extract, given by intramuscular injection, was a purified preparation containing 15 U.S.P. units per cubic centimeter. Thirty units were given in a single dose.

## RESULTS

The values obtained by the fractionation of urinary phenolic compounds under these experimental conditions are shown in Table I.

The daily values, before liver extract administration, for total phenols and for the various fractions show considerable variation among the different subjects but are more constant for each individual. The most constant value for each individual is the ratio of the ether-soluble-bicarbonate-soluble fraction to the ether-soluble-hydroxide-soluble fraction.

The results for the two ether-soluble phenol fractions are in fair agreement, both as to quantity and ratio, with those reported by Schmidt<sup>2</sup> who used another method.

At the beginning of the fifth day of the diet, 30 units of liver extract (2 c.c.) were given by intramuscular injection. In the subsequent twenty-four-hour period the excretion of total phenols was reduced in three subjects, remained the same in one subject, and was increased in one subject. The total phenols in most cases tended to increase for the remaining two days of the experimental period.

The excretion of ether-insoluble phenols is not affected by liver extract administration, hence changes in total phenols reflect changes in the ether-

<sup>1</sup>From The Thomas Henry Simpson Memorial Institute for Medical Research, University of Michigan.

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soluble phenols. When the two fractions of the ether-soluble phenols are considered separately, the effect of liver extract on their urinary excretion level is similar in all subjects. The bicarbonate-soluble fraction usually shows a considerable reduction, while the hydroxide fraction tends to be increased. As a result, the ratio of the bicarbonate-soluble fraction to the hydroxide-soluble fraction is significantly decreased.

The effect of this one dose of liver extract is transient, and the ratio of the ether-soluble phenolic components begins to increase again on the day following the liver extract supplementation.

TABLE I. FRACTIONATION OF URINARY PHENOLS IN NORMAL SUBJECTS; EFFECT OF A LIVER EXTRACT

SUBJECT	DAY ON DIET	ETHER-SOLUBLE PHENOLS		RATIO A:B (MG.)	ETHER- INSOLUBLE PHENOLS C (MG.)	TOTAL PHENOLS A + B + C (MG.)
		NaHCO <sub>3</sub> FRACT. A (MG.)	NaOH FRACT. B (MG.)			
SE F	3	127	70	1.8	40	237
	4	132	83	1.6	42	257
	5*	59	81	0.7	37	177
	6	70	56	1.3	46	172
	7	117	67	1.6	38	222
KE M	2	103	46	2.2	58	207
	3	105	48	2.2	64	217
	4	106	48	2.2	51	215
	5*	68	53	1.3	56	177
	6	70	48	1.5	51	169
	7	90	51	1.8	49	190
WA F	2	60	55	1.3	36	160
	3	77	62	1.2	38	177
	4	94	65	1.4	31	190
	5*	35	63	0.6	44	142
	6	60	85	0.7	38	183
	7	85	79	1.1	41	205
ST F	2	78	59	1.3	51	188
	3	72	49	1.5	46	167
	4	84	60	1.4	47	191
	5*	47	95	0.5	50	192
	6	59	81	0.7	48	188
	7	70	88	0.8	45	203
BR M	3	73	61	1.2	38	172
	4	82	70	1.2	42	194
	5*	80	98	0.8	44	222
	6	94	91	1.0	40	225
	7	104	82	1.1	38	224

\*On this day, 30 units of liver extract (15 units per cubic centimeter) were administered intramuscularly.

Values expressed as milligrams tyrosine excreted per twenty-four hours.

#### DISCUSSION

The ratio of the ether-soluble phenolic components stands out in this study of phenol excretion under controlled dietary conditions as the most constant value obtained. In other studies on the effects of various substances on phenolic excretion, it might therefore be well to give special consideration to the determination of changes in this ratio.

From the data in the preceding paper<sup>1</sup> it has been shown that the ratio of the ether-soluble phenolic compounds in pernicious anemia patients previous to treatment is much increased over that of the normal subjects. Therapy sharply reduces the ratio, but there is considerable day to day variation. The results of this study show that the administration of liver extract to a normal subject exerts a transient reduction in this ratio, but it is not reduced below the lowest ratio obtained in pernicious anemia in remission.

This transient effect of liver extract on the ratio of the ether-soluble phenolic components excreted by normal subjects may be taken as additional evidence that there is a substance present in liver that acts on the processes of phenolic oxidation either directly or indirectly. There are indications that this substance may be the liver antipernicious anemia principle.

The components of the weakly acidic phenolic fraction (phenol and *p*-cresol) are commonly regarded as products of enterogenous metabolism, which are absorbed from the gastrointestinal tract and excreted in the urine.<sup>3,4</sup> In both normal subjects and pernicious anemia patients there were instances where the excretion of this phenolic fraction was increased by liver extract injections. This increase would appear to be the result of an endogenous metabolic process, presumably an effect secondary to the increased oxidation of hydroxyphenyl acids.

#### SUMMARY

Values for the urinary excretion of various phenolic fractions from normal subjects on a regulated diet have been reported. The most constant value was the ratio for the ether-soluble phenols of the bicarbonate-soluble fraction to the hydroxide-soluble fraction.

When 30 units of liver extract were given, although the amount of total phenols excreted showed no consistent variation, there was a temporary reduction in the ratio of the ether-soluble phenolic components, due both to a decrease in the bicarbonate-soluble fraction containing the hydroxyphenyl acids and an increase in the hydroxide-soluble fraction.

#### REFERENCES

1. Swendseid, M. E., Wandruff, B., and Bethell, F. H.: Urinary Phenols in Pernicious Anemia, *J. Lab. & Clin. Med.* 32: 1242, 1947.
2. Schmidt, E. G.: Ether Extraction Method for Determination of Urine Phenols, *J. Biol. Chem.* 145: 533, 1942.
3. Baumann, E.: Weitere Beiträge zur Kenntniss der aromatischen Substanzen des Thierkörpers, *Ztschr. f. Physiol. Chem.* 4: 304, 1880.
4. Mitchell, H. H., and Hamilton, T. S.: Biochemistry of the Amino Acids, New York, 1929, The Chemical Catalog Company, p. 424.

# THE POTENCY OF THE U. S. P. REFERENCE STANDARD FOR VITAMIN D

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ARNOLD<sup>1</sup> has recently published data which indicate that calciferol (vitamin D<sub>2</sub>) has a potency of  $49.7 \pm 2.1$  U. S. P. units per microgram in terms of the present U. S. P. reference standard for vitamin D. From this he concludes that the "U. S. P. reference cod liver oil No. 2 has less than its generally accepted potency." If this conclusion is found to be valid, medicinal vitamin D preparations which are assayed by the U. S. P. technique must also be lower in potency than the assays indicate.

Fritz, Hooper, and Moore,<sup>2</sup> in connection with the Association of Official Agricultural Chemists' chick assay of vitamin D<sub>3</sub>, have previously called attention to the possibility that the U. S. P. reference standard may be deteriorating. In this connection it may also be pertinent to call attention to the fact that the vitamin A potency of the U. S. P. reference cod-liver oil No. 2 as determined by Callison and Orent-Keiles<sup>3</sup> was lower than its originally assigned value.<sup>4</sup>

Gridgeman<sup>5, 6</sup> has reported the potency of vitamin D<sub>2</sub> to be 36 I.U. per microgram and that of vitamin D<sub>3</sub> to be 46.4 I.U. per microgram. Although Gridgeman compared calciferol to the international standard, while Arnold<sup>1</sup> used the U. S. P. reference standard, Gridgeman's values strike a discordant note, since an overwhelming weight of evidence indicates that vitamin D<sub>2</sub> and D<sub>3</sub> have equal antirachitic activity for the rat. Remp and Marshall,<sup>7</sup> for instance, found vitamins D<sub>2</sub> and D<sub>3</sub> to be equal in potency for the rat. The results of a collaborative study in which nine laboratories participated were reported by Coward<sup>8</sup> and showed vitamins D<sub>2</sub> and D<sub>3</sub> to have equal potency for the rat. More recently, Huber and Barlow<sup>9</sup> have stated emphatically that the two vitamins behave exactly alike as to potency in the rat.

In 1943 Huber and Barlow<sup>9</sup> reported that, on the basis of over 400 satisfactory assays, the antirachitic value of calciferol was well over 40 U. S. P. units per microgram. Some time ago we also observed that samples of calciferol, when compared with the U. S. P. reference standard, consistently had an apparent potency at least 10 to 20 per cent higher than 40 U. S. P. units per microgram. We have therefore repeatedly assayed fresh samples of calciferol from three different sources against the U. S. P. reference cod-liver oil No. 2. The calciferol was dissolved in corn oil and assayed on rats of the Sprague-Dawley strain by the U. S. P. technique.<sup>10</sup> The results are given in Table I.

The initial assays seemed to indicate that the potency of the isolated sample was higher than that of the others. All subsequent assays, however, indicate that the potency of each of the three samples was over 48 and under 52 units per microgram.

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TABLE I. POTENCIES OF CALCIFEROLS

SOURCE OF CALCIFEROL	M.P. (CORR.) ° C.	n <sub>D</sub> 1% 1 CM. AT 265 mμ	[α] <sub>D</sub> <sup>25</sup> IN ETHANOL	APPARENT POTENCY (U. S. P. UNITS PER μg)
Winthrop Chemical Co.	115-117	465	+ 104.5 (c = .978)	50 ± 2
Glaxo Laboratories, Ltd.	115-117	460	+ 104 (c = 1.018)	50 ± 2
Isolated*	116-117.5	490	+ 103.7 (c = .982)	50 ± 2

C, grams per 100 milliliters.

\*Isolated from ergosterol activated by the Whittier Process.<sup>11</sup>

## DISCUSSION AND CONCLUSIONS

The antirachitic potency of the U. S. P. reference cod-liver oil No. 2 was originally determined by assay against the international standard for vitamin D.<sup>4</sup> The U. S. P. unit of vitamin D was, in fact, defined as "equal, in antirachitic potency for the rat, to one International Unit of Vitamin D as defined and adopted by the Conference of Vitamin Standards of the Permanent Commission on Biological Standardization of the League of Nations in June of 1931."<sup>12</sup> The antirachitic potency of pure calciferol, therefore, should be the same whether expressed in international units or in U. S. P. units. This appears not to be the case, at least when the U. S. P. standard currently being distributed is used.

The potency of calciferol is recorded in the report of the Second Conference on Vitamin Standardization of the League of Nations<sup>13</sup> as 40 I.U. per microgram. This value was confirmed in a comprehensive study by Anderson, Bacharach, and Smith<sup>14</sup> and by the collaborative study reported by Coward.<sup>8</sup>

Older values obtained by comparative assays against an earlier U. S. P. reference oil<sup>7, 15</sup> indicate that the potency of calciferol at that time was also 40 U. S. P. units per microgram in terms of the then current U. S. P. standard.

The discrepancy between the more recent and the older values for the potency of calciferol might be explained by the assumption that the samples of calciferol more recently available were of a higher degree of purity than the samples with which the older work was done. When the constants of the calciferol used in the aforementioned collaborative study,<sup>8</sup> of the calciferols studied by Anderson, Bacharach, and Smith,<sup>14</sup> and of the "pure calciferol" for which specifications were given in the report of the Second International Vitamin Conference<sup>13</sup> are compared with the constants of the samples of calciferol used by Huber and Barlow,<sup>9</sup> Arnold,<sup>1</sup> and ourselves, it appears that the earlier samples of calciferol were equally as pure as those used more recently.

The only reasonable explanation for the findings of Huber and Barlow,<sup>9</sup> Arnold,<sup>1</sup> and ourselves, therefore, appears to be that the U. S. P. reference standard for vitamin D is below its stated potency. These results and the findings of Callison and Orent-Keiles<sup>3</sup> with relation to the vitamin A potency of the standard strengthen the hypothesis of Fritz and co-workers<sup>2</sup> that the reference standard is deteriorating. It may therefore be advisable to re-evaluate the potency of the U. S. P. reference standard for vitamin D and to re-examine

recent data obtained by comparative assays with this standard. The substitution of a primary standard for the cod-liver oil standard should be considered. We concur in the recommendations of Arnold<sup>1</sup> and of Waddell and Kennedy<sup>16</sup> that pure, crystalline vitamin D<sub>3</sub> be adopted as the reference standard for vitamin D preparations.

## REFERENCES

1. Arnold, A.: The Biological Activity of Calciferol (Vitamin D<sub>2</sub>), *Proc. Soc. Exper. Biol. & Med.* 63: 230, 1946.
2. Fritz, J. C., Hooper, J. H., and Moore, H. P.: Calcification in the Poults, *Poultry Sci.* 24: 324, 1945.
3. Callison, E. C., and Orent-Keiles, E.: Standards in Vitamin A Assays. U. S. P. Reference Cod Liver Oil vs. Beta-Carotene, *Indust. & Engin. Chem. (Anal. Ed.)* 17: 378, 1945.
4. U. S. P. Advisory Board: The Preparation and Vitamin A and Vitamin D Standardization of the Second U. S. P. Reference Cod Liver Oil, *J. Am. Pharm. A. (Scient. Ed.)* 31: 124, 1942.
5. Gridgeman, N. T.: Special Designs for Vitamin D Assays, *Quart. J. Pharm. & Pharmacol.* 18: 15, 1945.
6. Gridgeman, N. T.: The Potencies of Vitamins D<sub>2</sub> and D<sub>3</sub>, *Quart. J. Pharm. & Pharmacol.* 18: 24, 1945.
7. Remp, D. G., and Marshall, I. H.: The Antirachitic Activity of Various Forms of Vitamin D in the Chick, *J. Nutrition* 15: 528, 1938.
8. Coward, K. H.: The Relative Antirachitic Potencies of Vitamin D<sub>2</sub> (Calciferol From Irradiated Ergosterol) and of Vitamin D<sub>3</sub> (From Irradiated 7-Dehydrocholesterol), *Bull. Health Organ., League of Nations* 9: 425, 1940-1941.
9. Huber, W., and Barlow, O. W.: Chemical and Biological Stability of Crystalline Vitamins D<sub>2</sub> and D<sub>3</sub> and Their Derivatives, *J. Biol. Chem.* 149: 125, 1943.
10. U. S. Pharmacopoeia XII, Easton, 1942, Mack Publishing Co., p. 635.
11. Dasler, W., and Bauer, C. D.: Electrically Activated Ergosterol (Whittier Process), *J. Biol. Chem.* 167: 581, 1947.
12. U. S. Pharmacopoeia XI, Easton, 1936, Mack Publishing Co., p. 261.
13. Second Conference of Vitamin Standardization, *Bull. Health Organ., League of Nations* 3: 428, 1934.
14. Anderson, F. W., Bacharach, A. L., Smith, E. L.: The Properties of Calciferol, *Analyst* 62: 430, 1937.
15. Dasler, W., and Steenbock, H.: Unpublished data, 1937-8.
16. Waddell, J., and Kennedy, G. H.: Antirachitic Potency of Pure Crystalline Vitamin D<sub>3</sub> in Comparison With the U. S. P. Reference Cod Liver Oil When Assayed by the Chick Method, *J. Assoc. Official Agr. Chem.* 30: 190, 1947.

# EFFECT OF TYROSINE, TRYPTOPHANE, AND THIOURACIL ON MELANURIA

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THERE has been much speculation on the origin of melanin and melanogen, pigments widely distributed in nature and found in pigmented tumors. Eppinger<sup>1</sup> concluded from feeding experiments with tryptophane and tyrosine in a case of melanosarcoma that tryptophane was the precursor of melanogen. Raper<sup>2</sup> demonstrated that tyrosine was converted to melanin by tyrosinase. Paschkis and co-workers<sup>3</sup> found that thiouracil inhibited the *in vitro* formation of melanin by tyrosinase.

The purpose of the present investigation was to repeat the experiments of Eppinger and to determine the effect of thiouracil upon the melanin metabolism of a 47-year-old white woman with a melanosarcoma presumably arising from the adrenal gland and metastasizing throughout the body, as revealed by post-mortem examination. In the course of these studies, an improved method for the quantitative determination of urinary melanogen was developed.

## METHODS

The urinary melanogen was determined as follows (modification of Thormählen reaction<sup>4</sup>):

To 5 c.c. of urine, 5 c.c. of a 33 $\frac{1}{3}$  per cent (w/v) solution of sodium nitroprusside were added, followed by 5 c.c. of a 10 per cent (w/v) sodium hydroxide solution, and then by 5 c.c. of a 33 $\frac{1}{3}$  per cent solution of acetic acid. The solution was made up to 500 c.c. with water. Five cubic centimeters of a standard indole solution (containing 0.5 mg. per cubic centimeter) were treated similarly. An A. C. model Fisher electrophotometer with a 650 A filter (with a wave length range of 600 to 700 m $\mu$ ) was used for the colorimetric comparisons.

Calibration studies with aqueous indole solutions indicated a high degree of conformity of the Thormählen reaction with Beer's law (Fig. 1). Quantitative recovery of added indole from urine was close to 100 per cent in the range of the patient's urinary "melanogen" concentrations (35 to 50 mg. per cent), as shown in Table I.

TABLE I. URINARY INDOLE RECOVERY EXPERIMENTS

ACTUAL CONCENTRATION (MG. %)	OBSERVED CONCENTRATION (MG. %)	AVERAGE OBSERVED CONCENTRATION (MG. %)	PER CENT RECOVERY
15	16.5	16.6	110.7
	16.6		
30	33.4	32.9	109.6
	32.5		
50	49.2	48.4	96.8
	47.5		
65	64.1	63.6	97.9
	63.0		

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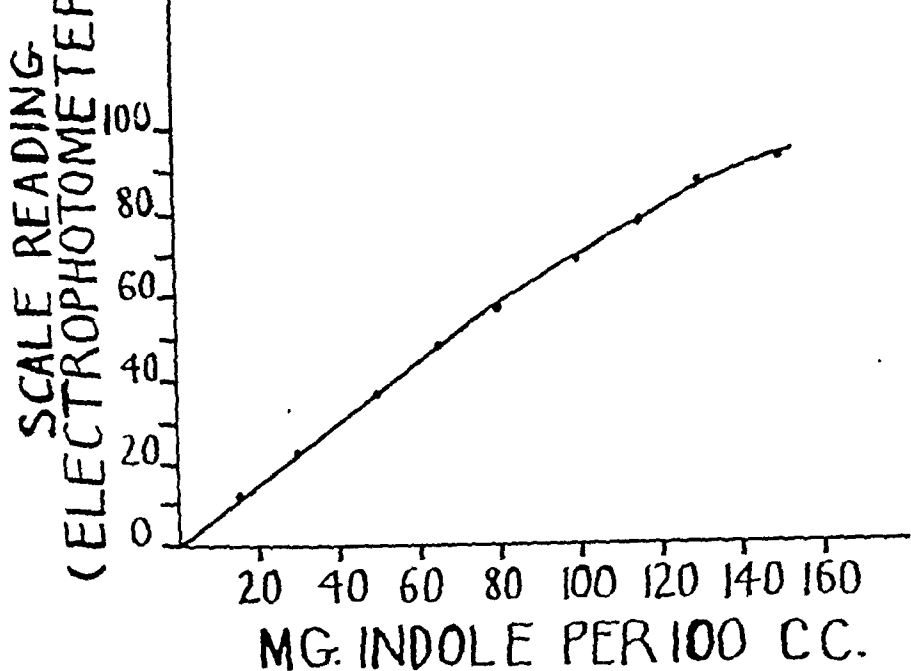


Fig. 1.—Electrophotometric scale reading (scale A) equals  $100 \times (2 - \log \text{ per cent transmission})$ .

A, C = 1.5 GMS. d-1-TRYPTOPHANE FED  
 B 2.0 GMS. l-TYROSINE FED

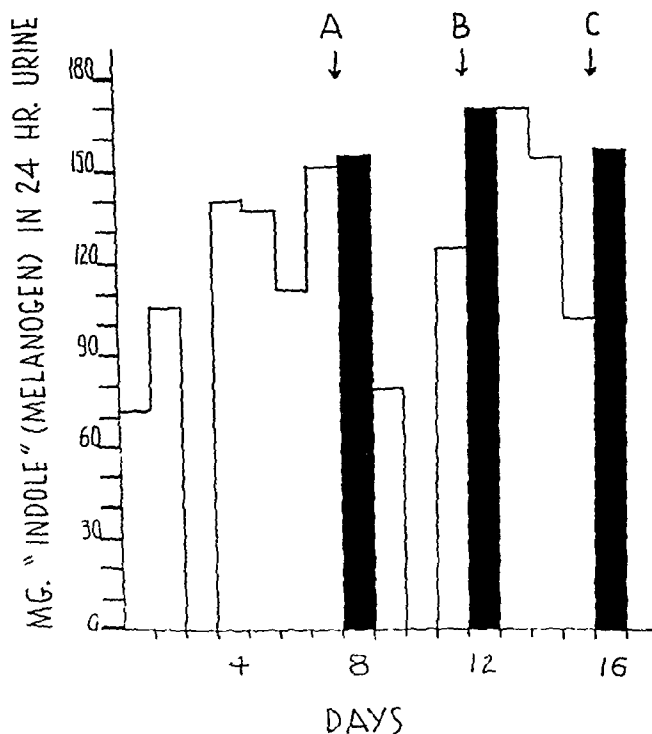


Fig. 2.—The amino acids were fed at A, B, and C at the beginning of the respective twenty-four hour periods. No melanogen determinations were performed on the third and tenth days.

Rothman<sup>5</sup> reported that the Thormählen test is negative in indicanuria. I have also observed one subject with indicanuria whose urine yielded a negative nitroprusside reaction. Indican, therefore, is not likely to be confused with melanogen.

#### EXPERIMENTAL

Our patient was fed 1.5 Gm. of dl-tryptophane, on each of two days, and 2.0 Gm. of l-tyrosine, on another day, as aqueous suspensions flavored with lemon juice.

The results are presented in Fig. 2.

In control studies of twenty-four hour urine specimens collected from ten hospital patients without melanomas and on a regular hospital diet, the Thormählen reaction was negative. In addition, random urines from about 100 patients with diverse diseases yielded a negative Thormählen reaction. Urines collected during the twenty-four hours following the feeding of 1.5 Gm. of dl-tryptophane to each of five normal men and 2.0 Gm. of l-tyrosine to each of five others failed in all cases to give the Thormählen reaction.

After the tyrosine and tryptophane feeding experiments were completed, 0.2 Gm. of thiouracil was administered to our patient three times a day. On the sixth day of drug administration the urine, which had previously been brownish-black, began to lighten in color, and by the eighth day the color approximated that of normal urine. Daily observations were made on each sample for one week or more. These normally colored urines did not darken through the week or more of observation after voiding, even though they were exposed to light (conditions under which melanogen is converted to melanin). The drug was discontinued on the tenth day. Three days thereafter the urine again became black. The patient died at this point, and the experiment could not be repeated.

Thiouracil did not affect the urinary color directly. When 0.2 Gm. of it (several hundredfold excess) was added to 10 c.c. of black urine and incubated at 37° C. for twenty-seven hours, no change in color was observed.

#### DISCUSSION

Investigators studying patients with melanosarcoma and melanuria have been limited by the fact that such patients do not live very long after the melanuria is detected; in this respect our patient was no exception.

In our patient both tyrosine and tryptophane increased the melanogen excretion slightly above the mean daily output of  $122.4 \pm 30.0$  mg., expressed as "indole," in the control period. After tryptophane was fed 154.9 and 156.4 mg. were excreted; after tyrosine, 169.4 milligrams. These results do not confirm the difference in the behavior of the two amino acids found by Eppinger. It should be noted that his patient was vomiting when she was fed tyrosine, which may account for his failure to observe any increase in the urinary melanogen excretion afterward.

A possible mechanism of thiouracil action in our patient is the inhibition of tyrosinase activity in the tumor cells.



Lynn and De Marie,<sup>6</sup> studying the melanophores of tadpoles, and Juhn,<sup>7</sup> the feathers of brown Leghorn capons, observed decreased pigmentation after thiouracil, but these reactions apparently did not involve the inhibition of tyrosinase.

After this study had been completed, Sugiura<sup>8</sup> reported that daily ingestion of 15 mg. of thiouracil by mice had no inhibitory effect on the growth of transplanted Harding-Passey mouse melanoma.

#### SUMMARY

1. The feeding of tyrosine and tryptophane to a patient with melanoma-sarcoma and melanuria resulted in a small, possibly significant, increase in the urinary excretion of melanogen.

2. Thiouracil administration caused the urine to change from black to a normal color.

3. The color reverted to black when the drug was discontinued.

4. A possible mechanism of the thiouracil action is the inhibition of melanin formation in the tumor cells.

I wish to extend my thanks for the advice and encouragement given by Dr. Louis Leiter, Chief of the Medical Division, Montefiore Hospital.

#### REFERENCES

1. Eppinger, H.: Ueber Melanurie, *Biochem. Ztschr.*, Berl. 28: 181-192, 1910.
2. (a) Raper, H. S.: XIV. The Tyrosinase-Tyrosine Reaction. VI. Production From Tyrosine of 5:6 Dihydroxyindole and 5:6:2-Carboxylic Acid—the Precursors of Melanin, *Biochem. J.* 21: 89-96, 1927.
- (b) Linnell, L., and Raper, H. S.: The Chromogen of Melanuria, *Biochem. J.* 29: 76-85, 1935.
3. Paschkis, K. E., Cantarow, A., Hart, W. M., and Rakoff, A. E.: Inhibitory Action of Thiouracil, Thiocarbamide and Other Compounds on Melanin Formation by Tyrosinase, *Proc. Soc. Exper. Biol. & Med.* 57: 37-39, 1944.
4. Thormählen, J.: Mittheilung über einen noch nicht bekannten Körper in pathologischem Menschenharn, *Virchow's Arch. f. path. Anat.* 108: 317-322, 1887.
5. Rothman, S.: Studies on Melanuria, *J. Lab. & Clin. Med.* 27: 687, 1942.
6. Lynn, W. G., and De Marie, Sr. A.: The Effect of Thiouracil Upon Pigmentation in the Tadpole, *Science* 104: 31, 1946.
7. (a) Juhn, Mary: The Effect of Thyroxin in the Thiouracil-Treated Brown Leghorn Capon, *Anat. Rec.* 89: 566, 1944.
- (b) Ibid: Effect of Thiouracil on Plumage of Brown Leghorn Capon, *Endocrinology* 35: 278-279, 1944.
8. Sugiura, K.: Conference on the Biology of Normal and Atypical Pigment Cell Growth, Nov. 15 and 16, 1946, New York Academy of Sciences, Section of Biology.

## ALLOXAN DIABETES IN THE MOUSE

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ALTHOUGH the effect of alloxan has been studied on a large number of species (rabbit,<sup>2, 6</sup> dog,<sup>5</sup> rat,<sup>5, 9</sup> monkey,<sup>3</sup> man,<sup>4</sup> frog,<sup>14</sup> and duck<sup>13</sup>), to date no reports have been noted on the production of diabetes in the mouse. Inasmuch as it is desirable to use a small diabetic animal when working with expensive materials, as would be the case with tracer substances, this study on mice was undertaken. We were interested in studying the effect of diphosphopyridine nucleotide (D.P.N.) on the action of alloxan and consequently turned to mice to conserve materials. Diphosphopyridine nucleotide and alloxan have been reported to compete with each other in a yeast apozymase system.<sup>10</sup> Since the alloxan inhibition of this enzyme system can be reversed by adding large quantities of diphosphopyridine nucleotide, it was thought desirable to test the effect of diphosphopyridine nucleotide on the production of diabetes with alloxan.

### METHOD

Alloxan monohydrate was injected intravenously into the tail vein of the mouse. The mouse was immobilized in a glass cylinder. The tail protruded through a notch in the rubber stopper used to plug one end. An air hole at the opposite end served for ventilation. The entire mouse was warmed on the heating coil of a 56° C. oven for one to several minutes. The induced vasodilatation in the tail facilitated the intravenous injection. A 26-gauge hypodermic needle was used, and blood was drawn into the syringe before starting the intravenous injection. The alloxan was injected as a 1 per cent solution in distilled water. Blood samples (0.05 c.c.) were removed forty-eight hours later by cutting the tip of the tail (after warming the mouse), and the sugar was determined by the Folin-Malmros micromethod.<sup>7</sup>

The diphosphopyridine nucleotide preparation\* used for the injections was only 20 per cent pure and was injected in doses of 750 mg. per kilogram intravenously. A separate vein was used for the alloxan which was injected two to five minutes following the diphosphopyridine nucleotide injection.

### DATA

Table I shows the effect of injecting various doses of alloxan intravenously in the mouse. The animals were allowed food and water, ad libitum, before and during the experiment. When no alloxan was administered the blood sugar levels of seven of eight mice varied between 169 and 180 mg. per 100 c.c.; only one mouse had a blood sugar value slightly greater than 200 mg. per 100 cubic centimeters. The average forty-eight hour blood sugar is tabulated for the various doses of alloxan used. The average forty-eight hour blood sugar values given in Table I also include those of animals which did not show any blood

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sugar response to alloxan. The incidence of diabetes can be judged by the number of mice with forty-eight hour blood sugar values greater than 250 mg. per 100 cubic centimeters. Mice with mild or questionable diabetes are included in the group whose forty-eight hour blood sugars are greater than 200 mg. per 100 cubic centimeters.

TABLE I. EFFECT OF ALLOXAN INJECTION (INTRAVENOUS) ON MICE

NUMBER OF MICE	DOSE OF ALLOXAN (MG./KG.)	AVERAGE 48-HOUR BLOOD SUGAR	% OF MICE WITH 48-HOUR BLOOD SUGAR (> 200 MG./100 C.C.)	% OF MICE WITH 48-HOUR BLOOD SUGAR (> 250 MG./100 C.C.)
8	0	178	12	0
2	30	203	50	0
7	40	358	71	43
4	50	372	100	50
8	75	464	100	88
2	100	600	100	100

At a dose of 40 mg. per kilogram, three of seven mice had forty-eight hour blood sugar values ranging between 504 and 644 mg. per 100 cubic centimeters. Two showed forty-eight hour blood sugar values slightly greater than 200 mg. per 100 c.c., and the remaining two showed forty-eight hour blood sugar values slightly under 200 mg. per 100 cubic centimeters. Clearly, then, only 43 per cent of seven animals developed significant diabetes at an alloxan dose of 40 mg. per kilogram. When the dose of alloxan was increased to 75 mg. per kilogram, seven of eight mice showed blood sugar values, at forty-eight hours, ranging between 436 and 596 mg. per 100 cubic centimeters. Only one of eight mice showed a blood sugar value at forty-eight hours of 240 mg. per 100 cubic centimeters. Thus, alloxan in doses of 75 mg. per kilogram produced diabetes in nearly all of the mice injected.

Two mice were injected intravenously with diphosphopyridine nucleotide in doses of 750 mg. per kilogram. Inasmuch as the diphosphopyridine nucleotide was only 20 per cent pure, this corresponds to a dose of 150 mg. per kilogram of pure diphosphopyridine nucleotide. Alloxan was injected in doses of 75 mg. per kilogram two to five minutes following the diphosphopyridine nucleotide administration, separate veins being used for each intravenous injection. Blood sugars were determined at forty-eight hours. The two animals receiving diphosphopyridine nucleotide plus alloxan had forty-eight hour blood sugars of 518 and 626 mg. per 100 cubic centimeters. Consequently, no effect could be attributed to the diphosphopyridine nucleotide injection.

#### DISCUSSION

It will be noted that mice respond to the intravenous injection of alloxan. The dose of alloxan required to produce diabetes in mice seems to be somewhat greater than that required for rats. For in the latter animal, the intravenous injection of alloxan in doses of 40 mg. per kilogram produced diabetes in 95 per cent of the animals injected.<sup>12</sup> As has been previously reported,<sup>12</sup> when alloxan is injected intravenously much smaller doses are required to produce a

given level of diabetes than is the case when the alloxan is injected intraperitoneally. This has also been found true in the mouse.

The amounts of diphosphopyridine nucleotide and alloxan used in these mouse experiments are of the same order of magnitude as the concentrations used by Kensler and co-workers<sup>10</sup> in studying a yeast apozymase system. In these latter experiments an apozymase preparation was suspended in buffer pH 6.5 in the presence of magnesium chloride, glucose, and hexose diphosphate. When the alloxan concentration was  $2.2 \times 10^{-4}$  molar and the diphosphopyridine nucleotide concentration was 15  $\mu\text{g}$  per 4 c.c., the enzyme activity was inhibited to the extent of 92 per cent. However, when the alloxan concentration was kept constant and the diphosphopyridine nucleotide concentration was increased to 525  $\mu\text{g}$  per 4 c.c., only 7 per cent inhibition took place. A diphosphopyridine nucleotide concentration of 525  $\mu\text{g}$  per 4 c.c. corresponds to a concentration of 133 mg. per liter, whereas an alloxan concentration of  $2.2 \times 10^{-4}$  molar corresponds to a concentration of 35 mg. per liter. In the mouse experiments the alloxan was injected in doses of 75 mg. per kilogram to insure diabetes. However, the concentration of alloxan that is actually acting on the mouse cannot be accurately determined. At the pH of the body (7.4), 50 per cent of the alloxan present is converted to a nondiabetogenic compound, alloxonic acid, within the first three minutes.<sup>11</sup> The rate of conversion at pH 6.5 (the conditions used in the apozymase experiments) would be expected to be much less, although the precise rate has not been determined. Alloxan is also reduced to dialuric acid by glutathione.<sup>1</sup> However, the rate of inactivation of alloxan by glutathione in the intact mouse or in the isolated apozymase system studied by Kensler and co-workers<sup>10</sup> cannot be accurately determined. Thus, although the conditions in the mouse experiments cannot be accurately compared with those used in the apozymase system, the amounts of alloxan and diphosphopyridine nucleotide used were of the same order of magnitude. The fact that no protection was observed cannot be taken as evidence that a competition between alloxan and diphosphopyridine nucleotide does not take place in the body, inasmuch as the latter compound may not be able to penetrate the cell membrane; although one might suspect that with beta cell degeneration taking place, the permeabilities might also be changed sufficiently to permit such a compound to penetrate. The presence of phosphatase in the blood may also have played a role in destroying the diphosphopyridine nucleotide administered.

#### SUMMARY

Alloxan, injected intravenously, produced diabetes in the mouse. With a dose of 75 mg. per kilogram seven of eight animals showed a forty-eight hour blood sugar value ranging between 436 and 596 mg. per 100 cubic centimeters. In two experiments using a large dose of diphosphopyridine nucleotide, injected immediately preceding a diabetogenic dose of alloxan, the course of the diabetes was not altered.

#### REFERENCES

1. Archibald, R. M.: Methods for the Determination of Alloxan Together With Observations of Certain Properties of Alloxan, *J. Biol. Chem.* 158: 347, 1945.
2. Bailey, C. C., and Bailey, O. T.: The Production of Diabetes Mellitus in Rabbits With Alloxan, *J. A. M. A.* 122: 1165, 1943.

3. Bajerjee, S.: Alloxan Diabetes in Monkeys, *Lancet* 247: 658, 1944.
4. Brunschwig, A., and Allen, J. G.: Specific Injurious Action of Alloxan Upon Pancreatic Islet Cells and Convoluted Tubules of the Kidney. Attempted Chemotherapy of Insulin-Producing Islet Cell Carcinoma in Man, *Cancer Research* 4: 45, 1944.
5. Dunn, J. S., and McLetchie, N. G. B.: Experimental Alloxan Diabetes in the Rat, *Lancet* 245: 384, 1943.
6. Dunn, J. S., Sheehan, H. L., and McLetchie, N. G. B.: Necrosis of the Islets of Langerhans Produced Experimentally, *Lancet* 244: 484, 1943.
7. Folin, O., and Malmros, H.: An Improved Form of Folin's Micro Method for Blood Sugar Determinations, *J. Biol. Chem.* 83: 115, 1929.
8. Goldner, M. G., and Gomori, G.: Alloxan Diabetes in the Dog, *Endocrinology* 33: 297, 1943.
9. Gomori, G., and Goldner, M. G.: Production of Diabetes Mellitus in Rats With Alloxan, *Proc. Soc. Exper. Biol. & Med.* 54: 287, 1943.
10. Kensler, C. J., Dexter, S. O., and Rhoads, C. P.: The Inhibition of a Diphosphopyridine Nucleotide System by Split Products of Dimethylaminobenzene, *Cancer Research* 2: 1, 1942.
11. Lazarow, A., and Levey, S.: *Unpublished data.*
12. Lazarow, A., and Palay, S. L.: The Production and Course of Alloxan Diabetes in the Rat, *J. LAB. & CLIN. MED.* 31: 1004, 1946.
13. Mirsky, I. Arthur: Alloxan Administration to the Duck, *Proc. Soc. Exper. Biol. & Med.* 59: 35, 1945.
14. Seiden, George: The Response of the Pancreatic Islands of the Frog (*Rana Pipiens*) to Alloxan, *Anat. Rec.* 91: 187, 1945.

# BACTEREMIA AND ACUTE MENINGITIS DUE TO *ALCALIGENES FAECALIS*

## A CASE REPORT

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*ALCALIGENES faecalis* is generally considered to be saprophytic and non-pathogenic. However, it seems well established that this organism can produce disease. A review of the literature reveals that *A. faecalis* has been associated with a variety of clinical conditions. Anderson<sup>1</sup> cultured it from the blood of two patients who exhibited a syndrome simulating rheumatic poly-arthritis. The organism was isolated from the blood of a patient with acute hepatitis and jaundice by McIntyre.<sup>2</sup> An *Alcaligenes* type of bacillus was cultured from the blood of a patient with a typhoidlike state by Hazen and Mortillaro,<sup>3</sup> and from the blood of three patients with gangrenous appendicitis by Weiss.<sup>4</sup> It was thought to be the cause of death in a patient with protracted sepsis reported on by Goldberg.<sup>5</sup> The organism has been cultured from gallstones<sup>6</sup> and renal calculi.<sup>7</sup> It has been considered the pathogenic agent in certain types of infant summer diarrhea<sup>8</sup> and the cause of a few cases of adult enterocolitis.<sup>9</sup>

There are only four cases reported in which *A. faecalis* was responsible for meningitis. Voorhies and Wilen<sup>10</sup> described a case with *A. faecalis* bacteremia and meningitis which apparently responded to sulfadiazine therapy. Spray and Hawk<sup>11</sup> reported a case of meningitis following otitis media. Mason<sup>12</sup> reported a meningitis complicating a meningocele, and Gatewood<sup>13</sup> described a case of meningitis following a craniotomy.

The case reported herein apparently represents the fifth case of meningitis due to *A. faecalis* to be described in medical literature.

## CASE REPORT

A. F. S., a 42-year-old merchant seaman, was admitted to the hospital on May 11, 1946, complaining of fever and weakness. He had been well until four days previously when he first noted intermittent periods of chilliness and fever. The following day he began to cough and noted general malaise and aching over the entire body. The symptoms gradually increased in severity and he vomited on two or three occasions. A few hours prior to admission he began perspiring profusely and noted extreme weakness. The remainder of the history was non-contributory.

*Physical Examination.*—On admission physical examination revealed a well-developed and well-nourished male who appeared prostrated. Temperature was 37.2° C. (99° F.); pulse 82; and blood pressure, 132/72. There was mild hyperemia of the pharynx and a few scattered crackling râles were heard over the lung fields.

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**Hospital Course.**—A tentative diagnosis of atypical pneumonia was made upon admission but the chest x-ray was clear. The patient was treated symptomatically but remained restless and complained of headache and some vertigo upon standing. On the third hospital day the headache became more intense, and slight rigidity of the neck was noted for the first time. The temperature which had been normal except during the first few hours in the hospital rose to 38° C. (100.5° F.). Lumbar puncture revealed a bloody fluid under 210 mm. of water pressure. It was not cultured. The patient was not relieved by the spinal puncture but continued to complain of severe headache and vomited frequently. The temperature, remained about 37.7° C. (100° F.).

On the fourth hospital day the spinal fluid was still bloody and under slightly increased pressure. The patient remained conscious and oriented but complained of headache almost continually and vomited frequently. On the fifth hospital day the laboratory reported growth of a motile gram-negative bacillus with slight tendency to bipolar staining. The patient was then started on streptomycin intramuscularly (2.0 Gm. daily divided into equal doses every three hours) and intrathecally (0.1 Gm. daily) and sulfadiazine. The blood sulfadiazine level was maintained about 10.0 mg. per cent. Streptomycin blood levels were not done.

The organism recovered from the spinal fluid on four successive days and from one blood culture was a motile gram-negative rod that did not produce acid or gas on carbohydrate media, produced an alkaline reaction in litmus milk, reduced nitrates to nitrites, and was indol negative. It was identified by our laboratory as *A. faecalis*. Subcultures from the spinal fluid and blood were also sent to the Bureau of Bacteriology of the Maryland State Department of Health and the identification of the organism was confirmed. The organism was not inhibited by 16 µg per milliliter of streptomycin or by 10 mg. per milliliter of sulfadiazine.

Following the institution of therapy with streptomycin and sulfadiazine the temperature rose over the next two days, reaching 39.5° C. (103.1° F.) on the seventh hospital day. The patient's general condition was otherwise unchanged. However, during this time the spinal fluid changed from bloody to cloudy fluid containing about 1,000 cells and cultures became negative for *A. faecalis*. A blood culture taken just before treatment was started was positive for *A. faecalis*. About the fifth day of treatment the temperature, which had been ranging about 39.5° C. (103.1° F.), began to drop by lysis. The headache decreased in intensity and the patient appeared greatly improved. The subsequent clinical course was one of gradual, progressive recovery.

The intrathecal streptomycin was discontinued after nine days but the intramuscular streptomycin was continued for thirteen days and the sulfadiazine for seventeen days.

Since no portal of entry of the infection was obvious, the patient was carefully surveyed prior to discharge from the hospital. Several routine urine analyses were negative and intravenous pyelogram was normal. *A. faecalis* could not be recovered from several stool cultures. Proctoscopic examination showed no abnormalities. Gastrointestinal series showed slight irregularity of the duodenal cap, but no ulcer could be identified. There was slight spasticity of the sigmoid colon on barium enema, but no diverticuli or other abnormalities could be found.

**Laboratory Findings.**—The most significant laboratory findings are shown in Fig. 1. Other findings were as follows: On admission the hemoglobin was 14.2 Gm.; white blood count, 16,200 per cubic millimeter with 69 per cent neutrophils (14 stabs), 3 per cent monocytes, 6 per cent eosinophiles, and 22 per cent lymphocytes. Blood Kline and Kahn tests were negative. Urinalysis showed an alkaline reaction and no significant findings. A heterophile antibody titer was 1:14. Agglutination titers were: typhoid "O," 1:20; typhoid "H," 1:80; paratyphoid A, 1:20; paratyphoid B, 1:40; *Brucella abortus*, negative; and proteus "OX19," negative.

Upon discharge the patient's white blood count was 9,200; hemoglobin, 13.2 Gm.; urine, negative; the spinal fluid and blood cultures showed no growth. Specific agglutinins for *A. faecalis* were not present in the blood at any time.

**Follow-up Examination.**—The patient was readmitted to the hospital on Nov. 2, 1946, for follow-up examination, approximately five months following discharge. At that time he stated that he was feeling well. He complained only of occasional frontal headaches and mild

vertigo. Complete physical examination, including detail neurological survey, was completely negative. Lumbar puncture revealed a clear fluid under normal pressure and with normal dynamics. The cell count was 0 and the globulin was negative and there was no growth on culture.

The patient was again observed on May 20, 1947, about one year after the illness. Complete neurological examination was again essentially normal.

DATE		5/11	5/12	5/13	5/14	5/15	5/16	5/17	5/18	5/19	5/20	5/21	5/22	5/23	5/24	5/25	5/26
TEMPERATURE	40°																
	39°																
	38°																
	37°																
STREPTOMYCIN INTRATHECALLY																	
STREPTOMYCIN INTRAMUSCULARLY																	
SULFADIAZINE																	
SULFADIAZINE IN WHOLE BLOOD MG. PER 100 CC.									13.2	8.1		9.0	6.5	7.1	14.1	14.3	11.4
BLOOD CULTURES																	
SPINAL FLUID	APPEARANCE	A. FAECALIS NO GROWTH															
		BLOODY															
		CLOUDY															
	TOTAL PROTEINS	CLEAR															
						69.2		78.0	63.0								
	SUGAR							32.4	46.8		36.0			43.2	56.3	73.8	
	GLOBULIN					4+	3+	2+									
	CELLS (W.B.C.)							565	253	585		1180	1216	660	470	320	7
	BACTERIA	A. FAECALIS															
		NO GROWTH															

Fig. 1.—Summary of pertinent clinical and laboratory data on a case of *A. faecalis* bacteremia and meningitis.

#### COMMENT

The isolation of *A. faecalis* in pure culture four times from the spinal fluid and once from the blood seems to establish a case of bacteremia and acute meningitis due to this organism. Though *A. faecalis* is fairly frequently encountered in stool cultures, it is generally not thought capable of producing disease. A few instances to the contrary have been cited herein. This case serves to illustrate that *A. faecalis* can produce disease under certain circumstances. Unfortunately we are unable to attribute this pathogenicity to a lack of resistance on the part of the host or to a proved increased virulence of this particular strain or organism. There was no known event to suggest a decrease in resistance of the host, and animal virulence tests were not done with the cultures of *A. faecalis*.

The part therapy played in the recovery of this patient is also uncertain. The in vitro sensitivity tests on the organism revealed that growth was not inhibited by 16  $\mu$ g per milliliter of streptomycin or by 10 mg. per milliliter of sulfadiazine. Both of these figures indicate considerable resistance on the part



of the organism, and generally one does not expect a favorable response to treatment under such circumstances. The slow clinical response in this case casts considerable doubt relative to the effect of either streptomycin or sulfadiazine. However, there may have been some synergistic activity between the two. One would not have expected recovery without some specific therapeutic effect, but spontaneous recovery cannot be disproved.

Textbooks of bacteriology express doubt relative to the production of specific agglutinins for *A. faecalis* in patients with infections due to this organism. However, Wyatt<sup>14</sup> and Stuart, Thompson, and Krikorian<sup>7</sup> have found agglutinins present in low titer. The case reported by Voorhies and Wilen<sup>10</sup> did not show agglutinins. They were not demonstrable in this case.

#### SUMMARY

A case of acute bacteremia and meningitis due to *A. faecalis* is described. Despite high in vitro resistance of the organism to both streptomycin and sulfadiazine alone, the patient recovered under treatment with these two agents; synergistic activity may have been present. No preceding infection or port of entry for *A. faecalis* could be demonstrated. Agglutinins for *A. faecalis* could not be demonstrated in the blood after recovery. Follow-up examinations five and twelve months after the onset of illness failed to reveal any abnormalities.

#### REFERENCES

1. Anderson, W. K.: Two Cases of Bacillus Alcaligenes Infection, Practitioner 131: 102, 1933.
2. McIntyre, C. A.: A Case of Blood Stream Infection With Faecalis Alcaligenes and Marked Hepatitis, Mil. Surgeon 79: 140, 1936.
3. Hazen, E. L., and Mortillaro, M.: Hitherto Undescribed Microorganism of Alcaligenes Group, J. LAB. & CLIN. MED. 21: 710, 1936.
4. Weiss, E.: Alcaligenes Organism From Blood of Three Patients With Gangrenous Appendicitis, J. Infect. Dis. 44: 394, 1929.
5. Goldberg, L. H.: Bacillus Alcaligenes Infections, New York State J. Med. 38: 122, 1938.
6. Daniel, W. W., and Greene, E. H.: Recovery of Bacillus Faecalis Alcaligenes From Gall Stones, South. M. J. 22: 977, 1929.
7. Stuart, G., Thompson, W. E., and Krikorian, K. S.: Bacillus Alcaligenes Faecalis as Organic Nucleus in Renal Calculus, Brit. J. Urol. 6: 243-7, 1934.
8. Webster, R.: Blood Culture in Summer Diarrhea, M. J. Australia 1: 460, 1919.
9. Shearman, C. H., and Moorhead, T. G.: Bacillaemia Due to Infection With B. Faecalis Alcaligenes, Brit. M. J. 2: 893, 1916.
10. Voorhies, Norton W., and Wilen, C. J.: Alcaligenes Faecalis Bacteremia, Am. J. M. Sc. 204: 719, 1942.
11. Spray, B. S., and Hawk, F. L.: Cerebrospinal Meningitis; Case Report With Unusual Bacteriological Findings, West Virginia M. J. 30: 564-565, 1934.
12. Mason, R. J.: Acute Meningitis Due to Bacillus Alcaligenes Faecalis, J. Pediat. 4: 514, 1934.
13. Gatewood, M. D.: Bacillus Faecalis Alcaligenes Meningitis, Am. J. Surg. 12: 435, 1931.
14. Wyatt, W. S.: Bacteremia Due to Bacillus Alcaligenes Faecalis, Am. J. M. Sc. 174: 181, 1927.

# LABORATORY METHODS

## THE THYMOL TEST OF MACLAGAN

STANDARDIZATION AND ADAPTATION TO THE EVELYN PHOTOELECTRIC COLORIMETER

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IN THE course of work with the thymol test of MacLagan,<sup>1, 2</sup> started early in 1945, it soon became apparent that the visual estimation of turbidity was not convenient for precise investigation.

Standards prepared according to King and Haslewood,<sup>3</sup> obtained from two different sources and also from our laboratory, were slightly different. Moreover, comparison in the case of lipemic or deeply icteric sera was difficult or impossible.

In an attempt to obviate these disadvantages, a blank prepared with serum and 0.85 per cent sodium chloride (1 to 60 volumes) was introduced for each determination. Although this modification made easier the measurement of the turbidity of sera with a high bilirubin or fat content, it did not eliminate the errors inherent to visual methods of nephelometric analysis.

In order to use the photoelectric colorimeter for the turbidimetric determinations, an easily reproducible and stable calibration was necessary. Serum plus sulfosalicylic acid and barium sulfate were found unsuitable as standards, due to great variation in the results obtained. Finally a copper sulfate solution was adopted for calibration; it is easily prepared and gives in different experiments almost identical readings at 660  $\mu$ .

In this study the Evelyn photoelectric colorimeter has been used.

### EXPERIMENTAL

#### *Choice of Filter.—*

Measurements at 660  $\mu$  are very convenient, due to little interference with bilirubin and hemoglobin light absorptions and due to enough sensitivity for turbidity. Shank and Hoagland<sup>4</sup> have emphasized these points.

#### *Calibration.—*

*Serum With Known Protein Content:* The turbidity developed when 3 per cent sulfosalicylic acid is added to diluted serum is the method recommended to check the gelatin standards in the thymol test.<sup>2</sup>

Sera of known protein concentration (Kjeldahl in duplicate) were diluted with 0.85 per cent sodium chloride, so as to obtain solutions corresponding from

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8 to 200 mg. of protein per cent; that is to say, from 0.8 to 20 thymol units. Three volumes of 3 per cent sulfosalicylic acid were added to each tube, and the readings were made in the Evelyn photoelectric colorimeter with No. 660 filter.

The results obtained with different sera and also with the same serum at different moments were very variable, as shown in Fig. 1.

This inconsistency of the turbidimetric measurements of serum protein has also been demonstrated for different albumin preparations by Shank and Hoagland.<sup>4</sup>

### THYMOL UNITS

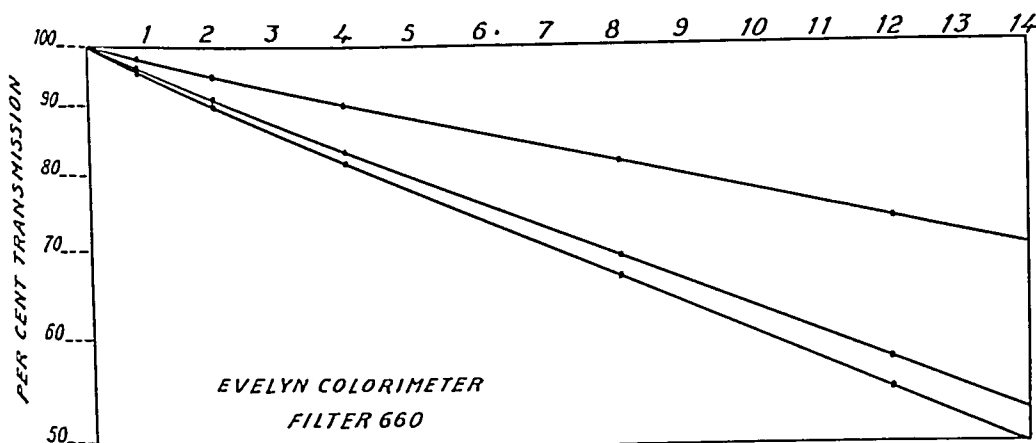


Fig. 1.—Calibration with diluted serum plus sulfosalicylic acid. Only three illustrative examples are reproduced. Values are expressed in thymol units (milligrams of protein divided by ten).

*Barium Sulfate:* Shank and Hoagland<sup>4</sup> have advocated a barium sulfate standard for the calibration of the spectrophotometer in the thymol test.

When this procedure was first employed very high values were found, but this was due to a typographic error in the original paper. The barium chloride solution is 0.0962 M (2 per cent) instead of 0.0962 N (1 per cent), according to the correction in the reprints.

Calibration with the barium sulfate standard is also variable, even if the same reagents and the same temperature are used, as shown in Fig. 2. This is probably due to different size of the precipitate particles and renders this method inconvenient for photoelectric calibration.

### Thymol Purity.—

The chemical purity of the thymol crystals has certain importance in the turbidity values obtained. Using two different brands, discrepancies were found in a great number of sera studied, as shown by the typical examples given in Table I. The reagents were prepared with the same chemicals and precautions, except for the thymol crystals.

### Blank.—

Even if the No. 660 filter is used in the photoelectric measurements, it is convenient to run a serum blank to eliminate the influence of natural opalescence,

TABLE I

SERUM	THYMOL CRYSTALS FROM	
	FRITZCHE BROTHERS, INC. (NEW YORK) (UNITS)	MERCK (DARMSTADT) (UNITS)
1	6.6	7.6
2	11.06	11.18
3	1.6	1.9
4	10.11	15.18
5	10.8	12.56
6	3.1	4.3
7	5.1	5.6
8	11.3	11.56

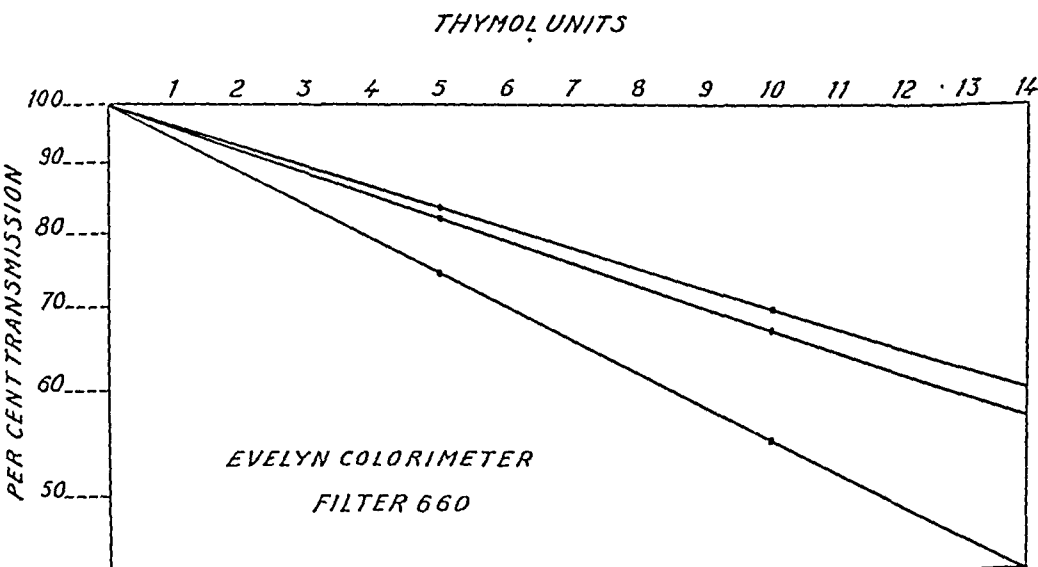


Fig. 2.—Calibration with barium sulfate. Only three illustrative examples are reproduced. Values are expressed according to Shank and Hoagland,<sup>4</sup> and in thymol units.

hemolysis, or high bilirubin content completely. This is shown in the typical examples given in Table II where readings using thymol buffer and serum plus saline as blanks, are compared.

The use of a serum-saline blank depends on having a clear thymol buffer. In our experience it has never become opalescent or turbid on standing, but this can happen under certain conditions apparently related to climate. Watson,<sup>5</sup> for instance, stated: "The thymol buffer solution is not as constant as one might wish; its turbidity varies a little from batch to batch, and from time to time. We find that on standing it becomes visibly more turbid over a period of weeks, and that this cannot be removed by filtration." Schloss<sup>6</sup> also refers to variations of the thymol reagent.

If one has to work with a more or less turbid thymol buffer, it is more convenient to use it as a blank instead of serum-saline.

After many trials, the following calibration has been adopted. It is very easy to perform and, although it is purely empirical, the values obtained are

TABLE II

SERUM	BLANK	
	THYMOL BUFFER (UNITS)	SERUM PLUS SALINE (UNITS)
1	4.3	5.1
2	2.96	1.39
3	2.13	2.5
4	6.2	5.1
5	12.06	11.56
6	3.2	2.89
7	3.5	2.8
8	5.0	3.8

similar to those reported by MacLagan with the visual estimation. A blue solution has been used since the readings for turbidity are taken with a red (No. 660) filter.

## METHOD

*Calibration.*—A 5 per cent aqueous solution of copper sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) is prepared and filtered if necessary. To 2 ml. in an Evelyn tube, 8.5 ml. of distilled water are added. From this diluted solution, 3 ml. and 1.5 ml. are placed in a second and third Evelyn tube, respectively, and the volume is taken up in both to 6 ml. with distilled water. The readings are made in the Evelyn colorimeter, using the No. 660 filter, the 6 c.c. aperture, and bright light, with distilled water as a blank. The equivalence of the standards in thymol units and a typical example of calibration are shown in Table III.

TABLE III

2 ML. OF 5 PER CENT $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ + 8.5 ML. OF DISTILLED WATER	DISTILLED WATER	UNITS	G
6 ml.	-	20	54'
3 ml.	3 ml.	10	73
1.5 ml.	4.5 ml.	5	85

The straight line relationship between the optical density of the standards is shown in Fig. 3. For convenience, a table with the correspondence between G values and thymol units is reproduced (Table IV).

The intensity of the blue color is not dependent upon the brand of copper sulfate or the time elapsed between preparation of the solution and colorimetric readings. Salts from three different sources (Baker; Schering, Kahlbaum; and Merck, Darmstadt) gave identical G values soon after preparation or after standing overnight at room temperature.

*Procedure.*—The thymol buffer is prepared as described by MacLagan,<sup>2</sup> using reagent grade thymol crystals. As the final pH of the solution somewhat varies, it is convenient to adjust it to 7.8. The buffer is stored at a temperature over 20° C., as recommended by MacLagan. On standing over a long period, the thymol solution may show a small amount of precipitate at the bottom of the flask; although it could still be used, it is better to discard it.

In each of two Evelyn tubes, 0.1 ml. of serum is measured; 6 ml. of thymol buffer and 6 ml. of 0.85 per cent sodium chloride are added, respectively. After

TABLE IV. EQUIVALENCE BETWEEN GALVANOMETER READINGS (G) IN THE EVELYN PHOTO-ELECTRIC COLORIMETER, AND THYMOL UNITS (FILTER No. 660, 6 c.c. APERTURE, AND BRIGHT LIGHT)

	0	1	2	3		0	1	2	3
99	0.4	0.33	0.23	0.19	73	9.9	9.8	9.7	9.6
98	0.7	0.63	0.56	0.49	72	10.3	10.2	10.1	10.0
97	1.0	0.93	0.86	0.79	71	10.8	10.68	10.56	10.44
96	1.3	1.23	1.16	1.09	70	11.3	11.18	11.06	10.94
95	1.6	1.53	1.46	1.39	69	11.8	11.68	11.56	11.44
94	1.9	1.83	1.76	1.69	68	12.3	12.18	12.06	11.94
93	2.2	2.13	2.06	1.99	67	12.8	12.68	12.56	12.44
92	2.5	2.43	2.36	2.29	66	13.3	13.18	13.06	12.94
91	2.8	2.73	2.66	2.59	65	13.8	13.68	13.56	13.44
90	3.1	3.03	2.96	2.89	64	14.3	14.18	14.06	13.94
89	3.5	3.4	3.3	3.2	63	14.8	14.68	14.56	14.44
88	3.9	3.8	3.7	3.6	62	15.3	15.18	15.06	14.94
87	4.3	4.2	4.1	4.0	61	15.8	15.68	15.56	15.44
86	4.7	4.6	4.5	4.4	60	16.4	16.25	16.1	15.95
85	5.1	5.0	4.9	4.8	59	17.0	16.85	16.7	16.55
84	5.5	5.4	5.3	5.2	58	17.6	17.45	17.3	17.15
83	5.9	5.8	5.7	5.6	57	18.2	18.05	17.9	17.75
82	6.3	6.2	6.1	6.0	56	18.8	18.65	18.5	18.35
81	6.7	6.6	6.5	6.4	55	19.4	19.25	19.1	18.95
80	7.1	7.0	6.9	6.8	54	20.0	19.85	19.7	19.55
79	7.5	7.4	7.3	7.2	53	20.6	20.45	20.3	20.15
78	7.9	7.8	7.7	7.6	52	21.2	21.05	20.9	20.75
77	8.3	8.2	8.1	8.0	51	21.9	21.73	21.56	21.39
76	8.7	8.6	8.5	8.4	50	22.6	22.43	22.26	22.09
75	9.1	9.0	8.9	8.8	49	23.3	23.13	22.96	22.79
74	9.5	9.4	9.3	9.2	48	24.0			

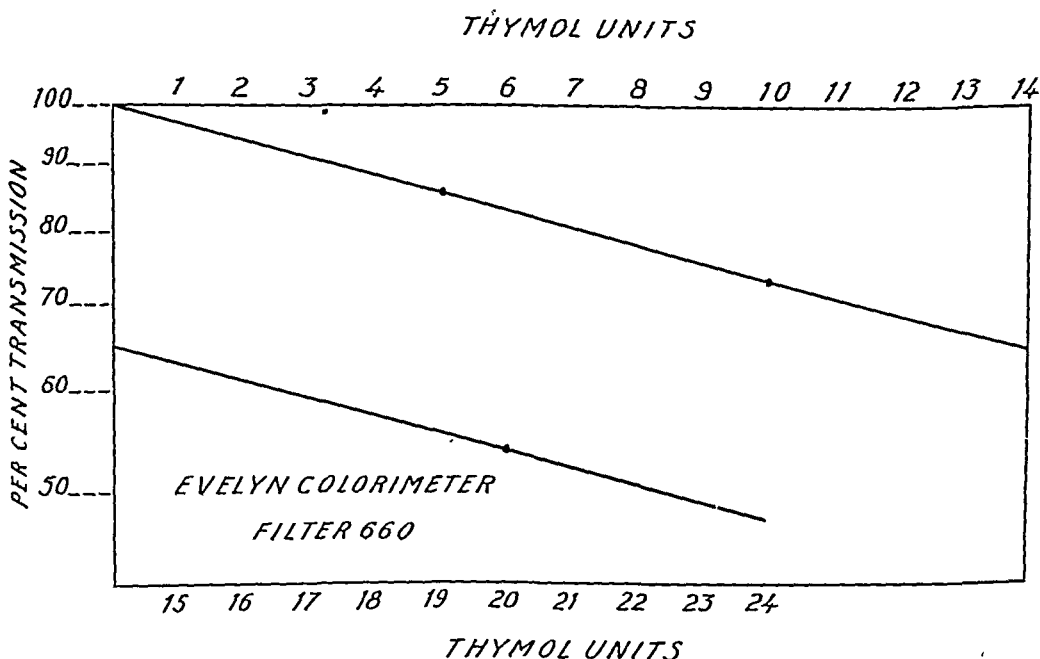


Fig. 3.—Calibration with copper sulfate. Values are expressed in thymol units.

standing for one-half to one hour, readings are taken in the Evelyn colorimeter (No. 660 filter, 6 c.e. aperture, and bright light), using the serum-saline tube as a blank. If the thymol solution is more or less turbid, a buffer blank is substituted for the serum-saline one. The value in thymol units is obtained from Table IV. It is convenient to shake the serum-thymol tube gently before placing it in the colorimeter, for some sedimentation may occur even in this short time.

After completion of the readings, the content of the serum-thymol tube is placed in a small Pyrex tube and left undisturbed overnight to allow observation of the amount of precipitate formed. This is graded from 0 (absent) to 3 plus; no 4 plus value has been used for some of the precipitate remains adherent to the walls of the tube, even with strongly positive sera.

#### NORMAL AND PATHOLOGIC VALUES

Using the method described, the range found in 100 apparently normal subjects was from 0.4 to 4.0 units.<sup>7, 8</sup> In only five of these cases some precipitate occurred after eighteen to twenty hours; four were recorded as 1 plus, and one as 2 plus.

In 152 of 170 cases of hepatocellular jaundice studied in the acute phase of the disease, the thymol turbidity test was over 4 units (89.4 per cent). Of the total, 111 were examples of infectious (sporadic) hepatitis, and fifty-nine, of cirrhosis with clinical icterus (thirty-four proved by punch biopsy, peritoneoscopy, or post-mortem examination). In 113 of these same cases (66.4 per cent) some degree of flocculation was observed.<sup>9</sup>

The thymol turbidity test was negative in twenty-two of thirty-five cases of obstructive jaundice (62.8 per cent) in which the diagnosis was confirmed by operation, necropsy, or punch biopsy; thirteen cases were due to cancer, nineteen to coledocolithiasis, and three to benign stricture of the common duct. Flocculation was negative in thirty; three of the positive results were 3 plus, and two, 2 plus.<sup>9</sup>

#### DISCUSSION

The thymol test of MacLagan is a useful complement to the laboratory study of diseases of the liver and bile ducts, as demonstrated by various reports.<sup>2, 4, 8-13</sup>

A drawback of the test is the visual estimation of turbidity advocated by MacLagan.<sup>1, 2</sup> Moreover, the gelatin standards of King and Haslewood<sup>3</sup> are not easy to prepare and vary in opalescence when they are obtained from different sources.

Albumin plus sulfosalicylic acid and barium sulfate have been demonstrated unsuitable for calibration on the colorimeter because they give inconsistent results.

Recently, Kunkel and Hoagland,<sup>14</sup> besides stating that the barium sulfate suspension has certain disadvantages, have advocated Evans blue dye (T-1824) as a standard of reference. Although this calibration is based on the same general principles of the one here presented, the concentration of 3  $\gamma$  per cubic centimeter (equivalent to 20 units) appears overestimated, for normal sera give values up to 8 units. It corresponds approximately to 10 units, using the copper sulfate solution as reference.

Ley and associates<sup>15</sup> have described an expression of the values for turbidity as cubic centimeters of barium chloride suspension. Besides being purely arbitrary, and based on barium sulfate obtained from 1 per cent barium chloride, it is subjected to the same criticisms as is Shank and Hoagland's calibration. There is no advantage in eliminating the original expression of results in units.

Additional data are needed to appreciate the value of the thymol flocculation. It has been less often positive, both in hepatocellular and obstructive jaundice, than the thymol turbidity. Apparently thymol flocculation is more useful in the follow-up of chronic hepatitis, as recently demonstrated by Neefe.<sup>16</sup>

#### SUMMARY

A modification of the thymol test of Maclagan is presented. The values for turbidity are obtained in the Evelyn photoelectric colorimeter, using No. 660 filter and a blank of serum plus physiologic saline. A buffer blank is employed when the thymol solution is more or less turbid.

The instrument is calibrated with a copper sulfate solution and the values expressed in units as originally advocated.

This calibration is easily performed, is independent of the salt brand, and is reproducible.

Flocculation is observed after eighteen to twenty hours and is expressed as 0, 1 plus, 2 plus, or 3 plus.

Normal values with this method are exactly those reported by the originator of the test.

#### REFERENCES

1. Maclagan, N. F.: Thymol Turbidity Test: A New Indicator of Liver Dysfunction, *Nature*, London 154: 670, 1944.
2. Maclagan, N. F.: The Thymol Turbidity Test as an Indicator of Liver Dysfunction, *Brit. J. Exper. Path.* 25: 234, 1944.
3. King, E. J., and Haslewood, G. A. D.: Permanent Standards for the Turbidometric Estimation of Protein, *Lancet* 2: 1153, 1936.
4. Shank, R. E., and Hoagland, C. L.: A Modified Method for the Quantitative Determination of the Thymol Turbidity Reaction of Serum, *J. Biol. Chem.* 162: 133, 1946.
5. Watson, C. J.: Personal communication.
6. Schloss, E. M.: The Applicability of Liver Function Tests in Jaundice of Acute Origin, *M. Clin. North America* 30: 1444, 1946.
7. Ducci, H., and Donoso, H.: Experiencia Clínica con la reacción del Oro Coloidal en el Suero, Especialmente en el Estudio de las Afecciones Hépatobiliares, Santiago, 1946, Stanley.
8. Ducci, H.: La Reacción del Timol de Maclagan; un Nuevo Aporte al Estudio de las Afecciones Hépatobiliares, *Rev. méd. de Chile* 75: 23, 1946.
9. Alessandri, H., Ducci, H., and Roeschmann, W.: Contribución del Laboratorio al Diagnóstico Diferencial de las Ictericias, *Rev. méd. de Chile* 75: 151, 1947.
10. Watson, C. J., and Rappaport, E. M.: A Comparison of the Results Obtained With the Hanger Cephalin-Cholesterol Flocculation Test and the Maclagan Thymol Turbidity Test in Patients With Liver Disease, *J. LAB. & CLIN. MED.* 30: 983, 1945.
11. Cohn, C., and Lidman, B.: Hepatitis Without Jaundice, in *Infectious Mononucleosis*, *J. Clin. Investigation* 25: 145, 1946.
12. Maclagan, N. F.: Thymol and Phosphatase in Jaundice, *Biochem. J.* 39: XXII, 1945.
13. Maizels, M.: Empirical Tests of Liver Function, *Lancet* 2: 451, 1946.
14. Kunkel, H. G., and Hoagland, C. L.: Persistence of Elevated Values for the Thymol Turbidity Test Following Infectious Hepatitis, *Proc. Soc. Exper. Biol. & Med.* 62: 258, 1946.
15. Ley, A. B., Lewis, J. H., and Davidson, C. S.: The Quantitative Determination of the Thymol Turbidity Reaction of Serum, *J. LAB. & CLIN. MED.* 31: 910, 1946.
16. Neefe, J. R.: Results of Hepatic Tests in Chronic Hepatitis Without Jaundice; Correlation With the Clinical Course and Liver Biopsy Findings, *Gastroenterology* 7: 1, 1946.



# THE COLLOIDAL RED TEST FOR THE STUDY OF HEPATIC DYSFUNCTION

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THE colloidal gold test, as modified by Maclagan,<sup>1</sup> is a valuable help in the laboratory study of hepatic disease.<sup>1-4</sup> Nevertheless, the preparation of the gold sol, used as a reagent in the test, has to be done with meticulous care, following many technical details, which renders it a cumbersome procedure.

Recently, Maizels<sup>5</sup> has suggested the use of a colloidal suspension of scarlet red which is added to three different dilutions of serum. In this test characteristic changes in color are observed in normal and in pathologic conditions. The main drawback of this reaction is the difficulty that arises in reading the results when icteric, hemolyzed, or opalescent sera are used.

After many trials, we<sup>6</sup> have adopted a test in which a modified red sol is used and which is carried out following the general principles of Maclagan's colloidal gold reaction. A buffer with a lower pH is used for the colloidal red suspension is more stable than the gold sol.

According to our present experience, the results of this test are the same as those obtained with Maclagan's colloidal gold reaction, and the red test has the advantages of being more reproducible and much simpler.

## COLLOIDAL RED TEST

### Reagents.—

1. Stock scarlet red solution: A saturated alcoholic solution of scarlet red (Scharlach R, Sudan IV)<sup>†</sup> is made by putting some of the powdered dye in a bottle and dissolving it in absolute ethyl alcohol. It is stored in the incubator at 37° C. at least for twenty-four hours before using. The solution must be saturated.

2. Buffer: (pH 7.53) 0.325 Gm. of barbital-sodium (sodium diethylbarbiturate;  $(C_2H_5)_2C:(CONa.CONH):CO$ ), 0.639 Gm. of barbital (acid diethylbarbituric;  $(C_2H_5)_2C:(CONH)_2:CO$ ) and 0.2 Gm. of phenol (carboic acid;  $C_6H_5OH$ ) are dissolved in 90 ml. of distilled water with the aid of heat, and after cooling, the volume is taken up to 100 milliliters. The pH is then checked and adjusted if necessary.

3. Scarlet red reagent: 10 ml. of the stock scarlet red solution in a 250 ml. Erlenmeyer flask and 50 ml. of distilled water in a 100 ml. Erlenmeyer flask are warmed to about 55° C. The water is then quickly added to the dye solution. The mixture is boiled first in the water bath and then over gauze till the final volume is about 20 milliliters. The volume is then made up to 800 ml. with distilled water. The reagent is kept in the refrigerator.

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<sup>†</sup>The "Harleco" dye, prepared by Hartman-Leddon Co., Philadelphia, Pa., has been found suitable for the test.

## Procedure.—

In a small Pyrex test tube, 0.05 ml. of serum and 0.5 ml. of buffer are placed. After mixing, 2.5 ml. of scarlet red reagent are added. The results are read, after the tubes have been left undisturbed at room temperature overnight, according to the following:

0 = No change

1 = Faint precipitate, visible only after shaking

4 = Supernatant fluid slightly colored, with heavy precipitate

5 = Clear supernatant fluid with heavy precipitate

(2 and 3 are intermediate grades of flocculation, mainly judged by the color of the supernatant fluid)

The data obtained by using this test, as compared with the cephalin flocculation, the colloidal gold test, the thymol test, the Takata-Ara reaction, and the Gros test, will be the subject of separate communications.<sup>7</sup>

## REFERENCES

1. MacLagan, N. F.: The Serum Colloidal Gold Reaction as a Liver Function Test, *Brit. J. Exper. Path.* 25: 15, 1944.
2. Alessandri, H., Ducci, H., and Donoso, H.: Experiencia Clínica con la Reacción del Oro Coloidal en el Suero, Especialmente en el Estudio de las Afecciones Hepatobiliares, *Día méd.* 18: 1495, 1946.
3. Armas, R., Cabello, J., Lobo, G., and Montero, E.: La Reacción del Oro Coloidal en el Suero Sanguíneo, con Especial Referencia a las Enfermedades hepatobiliares, *Rev. méd. de Chile* 74: 615, 1946.
4. Neeffe, J. R., and Reinhold, J. G.: Laboratory Aids in the Diagnosis and Management of Infectious (Epidemic) Hepatitis. Analysis of Results Obtained by Studies on 34 Volunteers During the Early and Convalescent Stages of Induced Hepatitis, *Gastroenterology* 7: 393, 1946.
5. Maizels, M.: Empirical Tests of Liver Function, *Lancet* 2: 451, 1946.
6. Ducci, H.: La reacción del Rojo Coloidal y la Adaptación al Colorímetro Fotoeléctrico de la Reacción de Enturbiamiento al Timol de MacLagan (Comunicación preliminar), *Rev. méd. de Chile* 74: 773, 1946.
7. Ducci, H., and Roeschmann, W.: The Value of the Flocculation Reactions in the Differential Diagnosis of Jaundice. To be published.

## PRODUCTION OF POTENT BLOOD GROUPING SERA

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THE procurement of potent blood grouping sera is a problem of importance to all those interested in research in blood groups and during World War II was also a source of concern to the military authorities. It was not until 1944 that Witebsky, Klendshoj, and McNeil<sup>7</sup> published a simple method which seemed to meet the demands. This involved the injection of small amounts of a mixture of A and B substances of animal origin into human volunteers of groups A and B. Since that time a number of workers<sup>2-4</sup> have shown the usefulness of the method.

The present paper is designed to report the results of another routine application of the method of Witebsky and co-workers<sup>7</sup> and to comment briefly on the safety and suitability of the procedure. From a group of medical students nine volunteers were selected and each given an intravenous injection of 0.9 c.c. of saline mixed with 0.1 c.c. of a solution of mixed A and B substance\* (7 mg. of A substance derived from hog stomach, and 5 mg. of A and B substance derived from horse stomach). No women were chosen because of the (very remote) possibility that the injection might at some later date favor sensitization to A or B factors during pregnancy,<sup>5</sup> but otherwise no selection was exercised, and the first ten men who volunteered were chosen. One of these later became apprehensive and dropped out of the experiment.

In my first use of these A and B materials, fear of an allergic type of reaction following the injection was not entirely absent from my mind at any time.<sup>2</sup> No such reaction has ever occurred, however, and it is interesting to record that Dr. S. B. Hooker, who before injection tested the present group of volunteers for skin sensitivity to the A and B materials as well as to pork and horse and human salivas of groups A and B, found nearly every individual to exhibit some skin reactivity to one or more of the test substances. One of the subjects exhibited a rather marked reaction to pork. Nevertheless, none of the subjects noticed any reaction, either immediate or delayed, following the injection. The danger from the procedure, judged by the total absence of symptoms in a large number of experiments in addition to those reported here, must be considered negligible.

The increases in isoagglutinin titer are shown in Table I. There was no difficulty, even with such a small group, in selecting from titrations of the trial bleedings one satisfactory A donor and one satisfactory B donor, which was all that was desired. From this and previous work the impression has been gained that about one-half of the volunteers respond with increases of titer and

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\*Supplied by Eli Lilly and Company, Indianapolis, Ind.

avidity sufficient to make their serum entirely satisfactory for use in routine blood grouping. In the present experiment the titer of the group B sera against  $A_2$  cells was not determined before the injection was made, since it was decided to use one of the donors in any case. The anti- $A_2$  titer which is shown by Subject 3, however, indicates that there had been a great increase in activity for this subgroup, and earlier work<sup>2</sup> shows that group B individuals, in fact, often show much greater relative increases in titer to  $A_2$  than to  $A_1$ , as well as great increases in avidity for  $A_2$ . This is one of the notable advantages of the Witebsky method of producing blood grouping sera, for reagents prepared in other ways often show inferior agglutinating power for the  $A_2$  antigen, especially when it is in combination with the antigen B in the subgroup  $A_2B$ .<sup>1</sup> Since many of the errors in grouping which are made in hospitals and in the Armed Forces probably result from a failure to recognize the presence of the antigen  $A_2$ , this is a point of considerable importance.

TABLE I. CHANGE IN TITER\* OF ISOAGGLUTININS TWO WEEKS AFTER AN INJECTION OF A AND B SUBSTANCE

SUBJECT	GROUP BLOOD	INITIAL TITER		FINAL TITER		
		$A_1$ CELLS	B CELLS	$A_1$ CELLS	$A_2$ CELLS	B CELLS
1	B	128	--	512	128	--
2	B	512	--	8192	512	--
3	B	512	--	2048	1024	--
4	A	--	32	--	--	256
5	A	--	64	--	--	128
6	A	--	64	--	--	256
7	A	--	32	--	--	64
8	A	--	16	--	--	512
9	A	--	8	--	--	16

\*Titer equals reciprocal of highest active dilution.

About 300 c.c. of blood were taken from Subject 2 and about 400 c.c. from Subject 8. The amounts of serum recovered from the clotted bloods were about 150 c.c. in each case. These sera seem to keep their activity indefinitely when kept frozen in the deep freeze.

It is interesting to speculate on the possible military applications of Witebsky's methods, in case large amounts of good blood grouping reagents should suddenly be desired in time of war. Suppose we have the problem of grouping 10,000,000 members of the Armed Forces. About twenty tests can be obtained from 1 c.c. of serum, but let us assume that we get only fifteen tests per cubic centimeter. Let us further assume that only one-half of our volunteers respond with the production of satisfactory isoagglutinins and that we obtain an average of 200 ml. of serum per volunteer. (Actual experience indicates that more serum than this can easily be obtained and, furthermore, that the agglutinin titer often remains high enough to enable successive bleedings to be taken at safe intervals. Moreover, many of the sera obtained are so potent that they can be diluted 1:4 or 1:8 with saline before use.) From these very conservative figures we can compute that  $2 \times 10,000,000 / (15 \times 200) = 6,666$  volunteers of group A and an equal number of group B will be required. This is a perfectly reasonable number of persons to deal with when one considers the large numbers that can be

vaccinated in a short time, and it is altogether probable that the favorable factors mentioned would reduce the number to little more than 1,000 volunteers of each group, with the result that a single organized center could produce enough blood grouping sera to determine the blood groups of the nation's entire Armed Forces. The expense would be largely confined to costs of personnel to give the injections, determine the final titers,\* and package the product. Aside from the packaging and distributing costs, the total expenses might be less than \$1,000.

#### SUMMARY

A routine use of the Witebsky method of producing potent grouping sera is described, and some considerations as to its suitability and low cost, in peace and in war, are offered.

#### REFERENCES

1. Boyd, W. C.: The Assay of Blood Grouping Sera: Variations in Reactivity of Cells of Different Individuals Belonging to Groups A and AB, *Ann. New York Acad. Sc.* 46: 927, 1946.
2. Boyd, W. C., and Lowell, F. C.: Specificity of Isoagglutinin Response Following Injection of Group Substances Into Group O Individuals, *Blood* 1: 548, 1946.
3. Diamond, L. K., and Boyd, W. C.: The Production of High-Titered Avid Human Iso-Immune A and B Grouping Sera, *New England J. Med.* (to be published).
4. Kabat, E. A.: Immunochemical Studies on Blood Groups. IV. Preparation of Blood Group A Substances From Human Sources and a Comparison of Their Chemical and Immunochemical Properties With Those of the Blood Group A Substance From Hog Stomach, *J. Exper. Med.* 85: 685, 1947.
5. Levine, P.: Genetic and Constitutional Causes of Fetal and Neonatal Morbidity, *Ann. New York Acad. Sc.* 46: 939, 1946.
6. Oncley, J., and others: Methods for the Preparation of Anti-A, Anti-B, and Anti-Rh Isoagglutinin Reagents, *Ann. New York Acad. Sc.* 46: 899, 1946.
7. Witebsky, E., Klendshoj, N. C., and McNeil, C.: Potent Typing Sera Produced by Treatment of Donors With Isolated Blood Group Specific Substances, *Proc. Soc. Exper. Biol. & Med.* 55: 167, 1944.

\*Actually, experience indicates that one of the important factors in the inferiority of blood grouping materials prepared in large batches is due to erroneous inclusion in a pool of serum<sup>6</sup> of the serum of one or more individuals who have been erroneously grouped, so it is probable that the best use of technical personnel would be to check the blood groups of each volunteer at least twice. Then the sera of all A volunteers and all B volunteers could be pooled, without doing titers. The sera of the subjects who failed to respond would simply dilute the strength of the final pool, probably by not over a factor of 2, which is easily allowable (see text).

# A SIMPLIFIED "QUALITATIVE" METHOD FOR HETEROPHILE ANTIBODY DETERMINATION USING CAPILLARY BLOOD AND A WHITE CELL PIPETTE

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SINCE the demonstration of a heterophile antibody in the serum of patients with infectious mononucleosis in 1932<sup>1</sup> many recognized methods for its determination have been published, all of which depend on the use of inactivated serum. An increasing interest in this disease and the recognition of ambulatory and asymptomatic forms<sup>2, 3</sup> suggested that a simplified "qualitative" test might be of value in clinic and survey work.

The purpose of this paper is to describe the technique of such a test, using capillary blood and a white cell pipette, and to report the results obtained with its use.

## EXPERIMENTAL CONSIDERATIONS AND PROCEDURES

For purposes of comparison with methods using serum for the determination of sheep cell agglutination, dilutions of whole blood must be considered in terms of the amount of heterophile antibody actually present. Since red cells occupy roughly one-half the volume of whole blood in normal individuals, two times any single dilution of whole blood would be the approximate equivalent of any similar dilution of plasma or serum alone in terms of the amount of heterophile antibody. The term equivalent titer will be used in this paper to express this relationship when whole blood dilutions are compared with serum dilutions. This same relationship should be true of most patients with infectious mononucleosis since this disease is rarely associated with an anemia.

Another factor bearing on the use of unheated whole blood for agglutination tests is the presence of hemolysins against sheep cells in normal human serum and in the serum of patients with infectious mononucleosis. Since actual demonstration of this capacity usually depends on the addition of complement as well as on incubation in a water bath, it was believed that less difficulty would be encountered from hemolysis if these procedures were avoided. With these considerations in mind, an experiment with whole blood was carried out to determine the practical applications of these concepts.

*Whole Blood Method.*—Serial twofold dilutions of citrated whole blood were made in saline solution in amounts of 1.0 ml. each. The tubes were centrifuged; 0.5 ml. of the supernate in each tube was mixed with an equal amount of 1 per cent suspension of sheep red blood cells and centrifuged at 2,000 r.p.m. for one to two minutes. The tubes were then shaken and the highest final dilution to show visible agglutination was taken as the end point; this was doubled to obtain titers comparable to dilutions of serum alone, and they were recorded as such.

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*Standard Method.*—The technique of Stuart<sup>1</sup> as modified by the Connecticut State Department of Health Laboratories was used. To twofold serial dilutions of inactivated serum in amounts of 0.5 ml. each, an equal volume of 1 per cent sheep red blood cell suspension was added; the tubes were incubated in a 37° C. water bath for two hours, and placed in the icebox overnight. Readings were made the following morning after shaking, and the highest final serum dilution showing visible agglutination was taken as the titer. Agglutination titers of 1:160 or more were considered as definitely elevated and are referred to as "positive" in this paper.

The results of simultaneous determination of sheep red blood cell agglutination titers by the methods outlined are listed in Table I for ten sera, five of which were from patients with proved infectious mononucleosis. It appears that the equivalent titers obtained by both methods were closely comparable. Furthermore, it should be recorded that no hemolysis of sheep red blood cells was observed in the experiments in which unheated whole blood was employed. These observations appeared to substantiate the theoretic considerations discussed previously and formed the experimental basis for the simplified test.

TABLE I. COMPARATIVE RESULTS OF SHEEP RED BLOOD CELL AGGLUTINATION TESTS WITH WHOLE BLOOD AND WITH INACTIVATED SERUM AS MEASURED IN TEN SAMPLES OF BLOOD

SAMPLE	SHEEP-RBC AGGLUTINATION TITER*	
	WHOLE BLOOD METHOD	STANDARD SERUM METHOD
1	20	10
2	20	40
3	10	10
4	40	40
5	80	80
6	160	160
7	640	640
8	640	640
9	640	640
10	1280	1280

\*Titer is expressed as reciprocal of serum dilution

#### THE SIMPLIFIED "QUALITATIVE" METHOD

1. Capillary blood was drawn up to the 0.5 mark in a standard white cell pipette and diluted to the 11 mark with normal saline.
2. This mixture was placed in a small test tube measuring approximately 7 cm. in length and with a diameter of 0.5 cm., and an equal volume of saline was added by refilling the white cell pipette to the 11 mark.
3. The tube was centrifuged at approximately 2,000 r.p.m. for one to two minutes.
4. The supernatant fluid was drawn up to the 11 mark on the white cell pipette and mixed with an equal volume of 1 per cent sheep red blood cell suspension in another small tube.
5. The test tube was centrifuged as before at 2,000 r.p.m. for one to two minutes and then shaken or struck sharply with the tips of the fingers until the sedimented cells became dislodged from the bottom of the tube. If definite evidence of agglutination was seen after thorough shaking, the test was recorded as "positive", if no agglutination was visible and the red blood cells had become resuspended, the test was recorded as "negative."

The term positive is used in the same sense as that just described under Standard Method, that is, a titer of sheep red blood cell agglutination considered unequivocally elevated, while the term negative includes all titers occurring within the normal range. The use of these terms appears justified since the equivalent titer used in the simplified test in these experiments actually corresponds to a dilution of serum of 1:160, previously taken as diagnostic in the standard method. It should be mentioned that a dilution equivalent to 1:80 can be obtained merely by drawing blood up to the 10 mark on the white cell pipette and proceeding as described.

While the designations positive and negative were always used in reporting results, a rough estimate of the actual titer in terms of serum dilution could be gained by observation of the type of agglutination: (1) High titer serum resulted in a tightly packed mass of agglutinated red blood cells that was dislodged easily from the bottom of the tube but tended to remain in large clumps on shaking; (2) titers in the low "positive" range of 1:160 and 1:320 resulted in more finely dispersed agglutinated particles; and (3) titers in the high normal range of 1:80 suggested agglutination when the tube was first agitated, but rapidly went into suspension.

Sheep red blood cell suspensions were often found suitable without rewashing for as long as five days if stored at icebox temperatures.

#### COMPARISON OF RESULTS OBTAINED WITH THE SIMPLIFIED AND THE STANDARD METHODS

One hundred twenty "qualitative" tests performed on capillary blood by the simplified method were checked by the standard method with serum obtained on the same day, and the results are listed in Table II. It will be seen that in no instance was there disagreement between the results obtained by the two methods: The results of ninety-four tests which were negative by the simplified method using capillary blood, when checked by the standard method using inactivated serum, showed titers that fell into the normal range. Similarly, all twenty-six positive tests using the simplified method were confirmed by finding significantly elevated sheep red blood cell agglutination titers by the standard method. It should also be noted that the results of the simplified test agreed with those of the standard method even at "borderline" titers of 1:80 and 1:160.

Eighty-four additional tests have been carried out by the simplified method. Of these, ten were found to be positive, and subsequent determination by the standard technique on the serum of seven of them confirmed these results. The remainder of the eighty-four tests were negative and were in agreement with the final diagnosis both from a clinical and a hematologic standpoint but were not checked by a standard quantitative method.

TABLE II. COMPARATIVE RESULTS OF SHEEP RED BLOOD CELL AGGLUTINATION TESTS

	STANDARD METHOD										SIMPLIFIED METHOD	
	NEGATIVE*					POSITIVE†					NEGATIVE	POSITIVE
TITER‡	<10	10	20	40	80	160	320	640	1280	2560		
Number of tests	20	29	23	15	7	3	3	11	7	2	94	26
Total tests			94					26			94	26

\*Negative = Sheep red blood cell agglutination titer within the normal range.

†Positive = Sheep red blood cell agglutination titer within the range diagnostic of infectious mononucleosis, namely, 160 or more.

‡Titer is expressed as reciprocal of serum dilution

#### DISCUSSION

The technique of a simplified "qualitative" method of determination of heterophile antibody has been described and the results obtained with this test have been confirmed with a standard quantitative method in 120 instances. The simplified method as used in these experiments was set up at a dilution equivalent to that of a 1:160 dilution of serum. While it has been stated that the presence of agglutination at this titer has been regarded as unequivocal evidence of a significantly elevated titer, it is evident that no precise dividing



point can actually be determined between the normal and the abnormal. It is felt, however, that agglutination at this titer occurs in a negligible number of normal human sera and can usually be accepted as diagnostic of infectious mononucleosis in the absence of recent serum injections. For those desiring a more sensitive test that might indicate early or convalescent cases of infectious mononucleosis, the simplified test is easily adjustable to the equivalent serum dilution of 1:80 as described under Method. Thus at the higher dilution the test can be used as a diagnostic tool and at the lower dilution for survey work during a suspected epidemic.

#### SUMMARY

1. Theoretic and practical considerations pertaining to the use of whole blood instead of inactivated serum for the determination of sheep red blood cell agglutination have been discussed and investigated.

2. A simplified "qualitative" method of heterophile antibody determination using capillary blood and a white cell pipette has been described.

3. Comparative results of 120 tests determined by the simplified method have been checked using a standard technique and no instance of disagreement found.

4. The simplified test is adaptable either to survey work or to diagnosis.

Appreciation is expressed to Dr. John S. Hathaway, Yale University Health Department, for suggesting the need for such a test as reported and for aid in collecting data, and to Miss Ruth Northey, of that same department, for technical assistance.

#### REFERENCES

1. Paul, J. R., and Bunnell, W. W.: Presence of Heterophile Antibodies in Infectious Mononucleosis, *Am. J. M. Sci.* 183: 90, 1932.
2. Vander Meer, R., Lutterloh, C. H., and Pilot, J.: Infectious Mononucleosis: An Analysis of 26 Clinical and 340 Subclinical Cases, *Am. J. M. Sci.* 210: 765, 1945.
3. Halcrow, J. P. A., Owen, L. M., and Roger, N. C.: Infectious Mononucleosis With an Account of an Epidemic Occurring in an E. M. S. Hospital, *Brit. M. J.* 2: 443, 1943.
4. American Public Health Association: Diagnostic Procedures and Reagents, New York, 1945, American Public Health Association Press, p. 449.

# A SIMPLE METHOD FOR THE DETERMINATION OF SALICYLATES IN BLOOD

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THE following is a rapid and simple method for determining salicylates in the blood. It is based on the use of the xanthoproteic reaction.

## TECHNIQUE

Mix thoroughly 1 ml. plasma or serum with 9 ml. trichloroacetic acid (7 per cent) and let stand fifteen minutes. Filter through a Whatman No. 5 paper (the filtrate must be absolutely clear). Deliver 2.5 ml. of filtrate into a test tube marked at 10 ml. and mix thoroughly with 1 ml.  $\text{HNO}_3$  (concentrated). Add distilled water to 5 ml. and mix again. Place in boiling water bath for five minutes. Cool in cold water bath. Add 3 ml.  $\text{NaOH}$  (60 per cent), mix thoroughly, and cool in cold water bath. Dilute to 10 ml. with water, mix, and read in colorimeter.

## RESULTS

The readings by means of the photoelectric colorimeter\* with filter No. 511† were plotted against the amounts of sodium salicylates dissolved in normal human plasma‡ (see Table I). Under these experimental conditions the intensity of the yellow color is found to be directly proportional to the concentration;

TABLE I. RECOVERY VALUES OF SODIUM SALICYLATES IN HUMAN PLASMA IN COMPARISON WITH THE THEORETIC READINGS

CONCENTRATION OF SALICYLATE C (MG. %)	COLORIMETER READING R	COLORIMETER READING R	COLORIMETER READING CORRESPONDING TO S C
5	39	39	40
10	80	80	80
15	120	120	120
20	161	161	160
25	200	200	200
30	236	240	240
35	273	273	280
40	314	324	320
45	358	353	360
50	404	407	400
55	449	449	440
60	484	478	480
70	566	566	560
80	646	646	640

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\*Klett-Summerson.

†Corning Glass Works.

‡Human sera contain small amounts of phenol derivatives which contribute to the xanthoproteic reaction. Although they do not correspond to more than 3 mg. per cent (except in cases of renal insufficiency, hepatitis, and a few rare conditions<sup>2</sup>), this factor was eliminated by preparing the curve with dilutions of salicylates in human serum.

in the present case, by a factor of 8. Thus R, the colorimeter reading, equals  $8 \times C$ , the concentration of salicylate in milligrams per 100 cubic centimeters.

A series of comparative determinations with our technique and that of Coburn<sup>1</sup> showed them to be in good agreement (see Table II).

TABLE II. COMPARISON OF VALUES BETWEEN XANTHOPROTEIC REACTION AND THE TECHNIQUE OF COBURN<sup>1</sup>

NO.	XANTHOPROTEIC	COBURN	DIFFERENCE (MG. %)
1	8.5	9.2	- 0.7
2	37.5	40.0	- 2.5
3	25.5	23.3	+ 2.2
4	15.0	15.0	0
5	7.5	7.9	- 0.4
6	12.0	11.7	+ 0.3
7	15.0	15.0	0
8	6.5	6.8	- 0.3
9	41.5	38.2	+ 3.3
10	15.0	13.5	+ 1.5
11	32.0	29.6	+ 2.4
12	39.0	37.2	+ 1.8
13	47.0	48.0	- 1.0
14	35.0	33.6	+ 1.4
15	32.0	32.0	0
16	37.5	40.6	- 3.1
17	38.5	34.0	+ 4.5
18	41.0	40.7	+ 0.3
19	33.0	30.6	+ 2.4
20	18.0	14.2	+ 3.8
21	55.0	49.0	+ 6.0
22	6.0	5.0	+ 1.0
23	30.0	30.8	- 0.8
24	29.0	32.6	- 3.6
25	11.5	10.0	+ 1.5

A series of standard potassium dichromate solutions may be used in place of the photoelectric colorimeter. The shade of the yellow color of diluted potassium dichromate perfectly matches that of the xanthoproteic reaction, as given by salicylate. These potassium dichromate solutions may be used either in the visual colorimeter or in sealed tubes to be matched directly by eye, as in the evaluation of the icterus index. For this purpose 4.08 Gm. of potassium dichromate are diluted to 1 liter to make a stock solution from which the series of standards is prepared. If the stock solution is diluted ten times, the resulting solution is equivalent to 60 mg. of salicylate. Further dilutions may be prepared for values down to 10 mg. per cent.

#### SUMMARY

A simple and rapid method for the determination of blood salicylates, based on the use of the xanthoproteic reaction, has been described.

#### REFERENCES

1. Coburn, A. F.: Salicylate Therapy in Rheumatic Fever, *Bull. Johns Hopkins Hosp.* 73: 435, 1943.
2. Volterra, M.: La Reazione Xantoproteica sul Siero e sugli Altri Liquidi Organici. Suo Determinismo Chimico e Valore Clinico, *Riv. di clin. med.* 29: 551, 1928.

# THE DETERMINATION OF THE HEART RATE IN THE RAT

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SEVERAL investigators have presented data regarding the heart rate in the rat. Woodbury and Hamilton<sup>1</sup> used the closed optical manometer to compare several species and reported an average rate of 420 per minute. A more usual procedure at present is to calculate the rate from the electrocardiographic tracing.<sup>2, 3</sup> This latter method has yielded rates averaging 430 per minute.

In view of the fact that these methods require a certain amount of manipulation of the animal, it appeared important to devise a method which would give absolute data for the rat at rest. If this could be achieved, other methods would then be amenable to interpretation. Such a method would be of still greater value in the experimental approach to cardiovascular disease if it recorded not only the rate of the heart, but gave some indication of the force and character of the beat as well. An electric apparatus was devised to meet these requirements.

## APPARATUS

The apparatus consists essentially of a crystal pickup arranged to receive impulses directly from the chest wall. The output is fed into a cathode ray oscilloscope. The pattern of the heart sounds appears on the screen and this is synchronized with the sweep of the time base. The time base is calibrated in cycles per minute so that when one complete heart pattern becomes stationary the heart rate corresponds with the repetition rate of the time base and is read directly from the dial.

*Pickup.*—For unanesthetized animals the stand and holder shown in Fig. 1 are used. The animal is placed in a cardboard holder of correct size in which an aperture large enough to expose the chest surface has been cut. The holder is placed on the carrier slings with the animal's chest directly over the curved brass chest plate. The carrier slings are racked up or down to bring the plate into contact with the chest. Contact should be firm but not constricting. The heart sounds are conveyed through the chest plate and its connecting arm into the crystal. The crystal used is an astatic cartridge type B2.

*Oscilloscope.*—Fig. 2 presents the wiring diagram for a three-inch cathode ray oscilloscope with its contained amplifier. This apparatus differs from the commercial varieties only in detail. The amplifier unit is designed to provide high gain with good low frequency response down to about 40 cycles per second, which is suitable for the sound frequencies encountered in this work. The time base is adapted for low sweep rates. The frequency dial is calibrated in cycles per minute, and the potentiometer control should have a linear taper to provide a linear scale. The circuit as designed covers the range of 180 to 720 cycles per minute, although switches conveniently extend this range in two further steps. A synchronization control is available to "lock" the heartbeat with the time base. To do this, the frequency rate must be reduced to approximately one-half that of the heart rate. The first heart cycle is used to trigger the time base and consequently does not appear in full on the screen. The following beat, however, appears in full and the pattern remains steady without the operator having to manipulate the frequency control. When this control is used, the frequency reading is meaningless.

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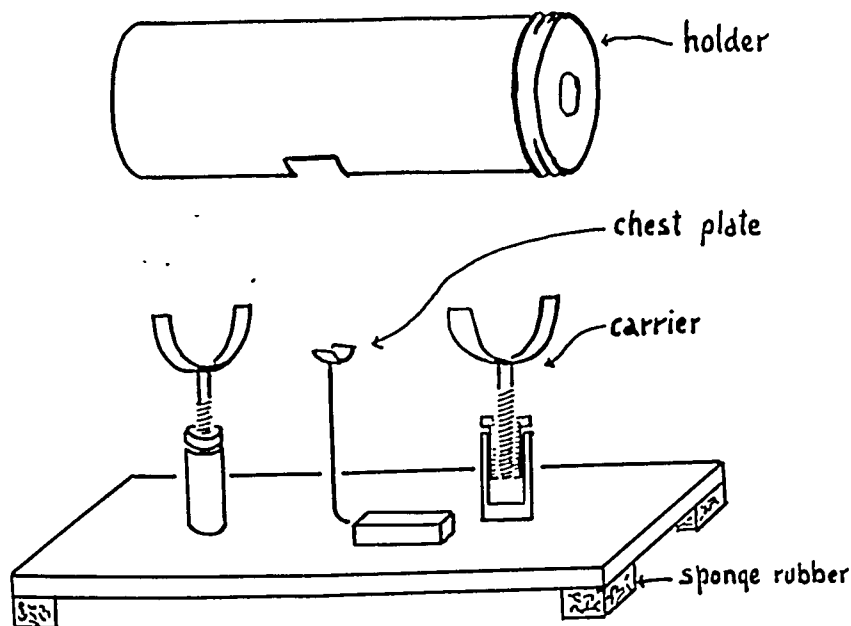
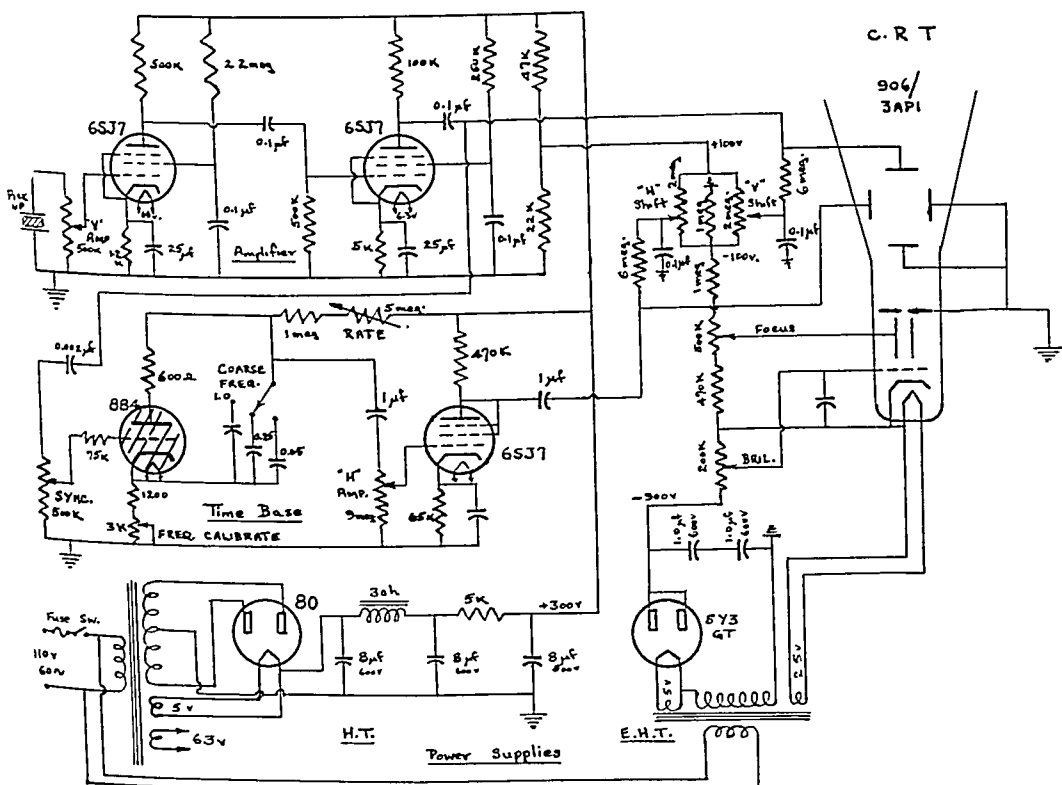


Fig. 1.



**Fig. 2.**

## EXPERIMENTAL

Data for the heart rate in the unanesthetized rat as well as in animals anesthetized with ether or nembutal are presented in Table I. The normal heart rate of  $310 \pm 30$  in trained unanesthetized animals averaging 150 grams in weight is lower than that previously reported. Nembutal depresses the rate only very slightly, while ether conspicuously increases it.

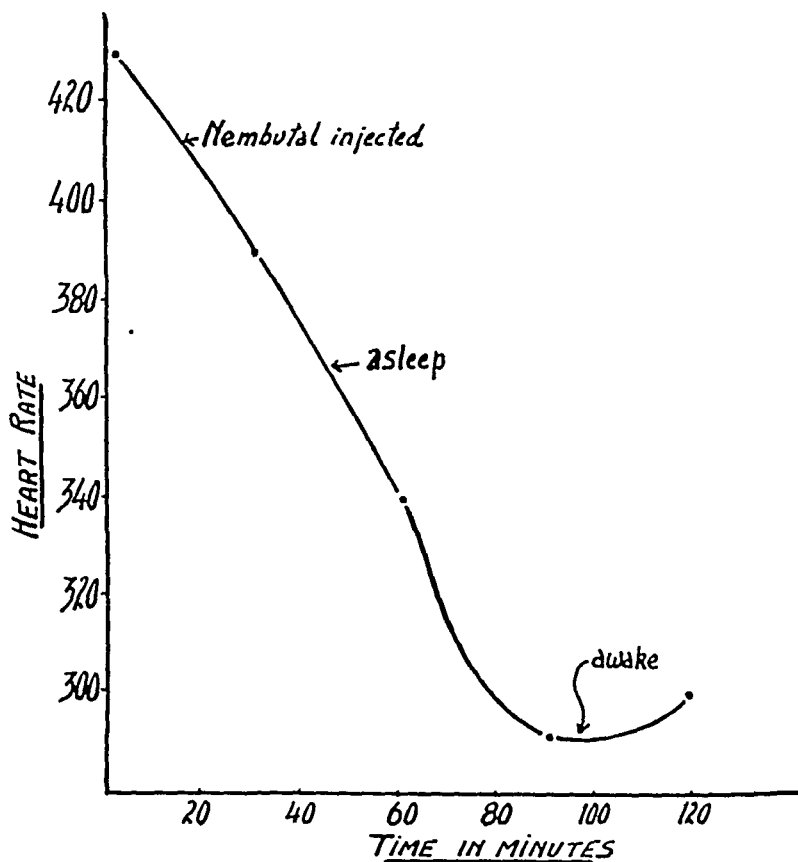


Fig. 3.

TABLE I

GROUP	NUMBER OF ANIMALS	CARDIAC RATE
Normal trained	22	$310 \pm 30$
Normal untrained	10	$480 \pm 20$
Ether		
Preanesthesia (trained)	5	300
Anesthesia	5	460
20 minutes postanesthesia	5	310
Nembutal		
Preanesthesia (partly trained)	6	350
60 minutes after injection	6	310
90 minutes after injection	6	310
120 minutes after injection (awake)	6	310

Subsequent work made it clear that the heart rate was extremely labile. A short stem chest contact was prepared and the rats held manually. The average rate for such manually held rats was 480 beats per minute. Indeed, in several such animals we were able to observe an increase from a starting rate of about 300 to more than 450 beats per minute in the space of thirty seconds. Similar results were obtained with animals in holders if they had not previously been trained. It was found that steady and reproducible results could be obtained by placing the animals in holders for one-half hour a day and determining the rate on the third day. To simplify this we use standard millboard mailing cartons with screw lids as holders. A hole for the tail is bored through the lid and an aperture for the chest contact cut in one side. Large groups can thus be handled since the actual determination of rate after training is a very brief procedure.

For most purposes nembutal given intraperitoneally in small doses is very satisfactory and avoids the training period without apparently affecting the heart rate appreciably. Fig. 3 shows the heart rate curve in an untrained animal following a light dose of nembutal. Nembutal is also advisable when the heart sounds are to be studied. The normal heart sound picture shows two characteristic low frequency patterns, one of greater amplitude than the other. These sounds are close together and occupy about one-half the duration of the cardiac cycle. Murmurs can readily be seen and interpreted.

#### SUMMARY

1. An apparatus for the determination of the heart rate in the rat has been described.
2. The normal heart rate in the unanesthetized rat averages 300 per minute.
3. The apparatus also makes possible the study of heart sounds in this species.

#### REFERENCES

1. Woodbury, R. A., and Hamilton, W. F.: Blood Pressure Studies in Small Animals, *Am. J. Physiol.* 119: 663-674, 1937.
2. Marcuse, F., and Moore, A. U.: Heart Rate and Respiration Preceding and Following Audiogenic Seizures in the White Rat, *Proc. Soc. Exper. Biol. & Med.* 48: 201-202, 1941.
3. Kellaway, P. E., Hoff, H. E., and Leblond, C. P.: The Response to Thyroxine After Subtotal Hepatectomy, *Endocrinology* 36: 272-279, 1945.

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#### Erratum

In the article by Pijper, entitled "The Diffraction Method of Measuring Red Blood Cells," in the July, 1947, issue of the JOURNAL, one of the color plates, Fig. 4, is printed upside down.

## BOOKS RECEIVED

- Physician's Handbook.** By *John Warkentin*, Ph.D., M.D.; and *J. D. Lange*, M.S., M.D. University Medical Publishers, Chicago, 1946. Fourth edition. Price \$1.50. Paper with 260 pages.
- Methods of Vitamin Assay.** Prepared and edited by the Association of Vitamin Chemists, Inc. Interscience Publishers, Inc., New York, 1947. Price \$3.50. Cloth with 181 pages.
- Histopathology of the Ear, Nose, and Throat.** By *A. A. Eggston*, B.S., M.D.; Director of Laboratories, Manhattan Eye, Ear and Throat Hospital; Clinical Professor of Pathology (Post Graduate Division) New York University Medical College; Director of Laboratories of the Mount Vernon Hospital; Pathologist, Harlem Eye and Ear Hospital; Diplomate of Pathology; formerly Associate in Pathology, Vanderbilt University Medical School, and College of Physician and Surgeons, Columbia University; and *D. Wolff*, A.B., M.A., Ph.D.; Research Investigator, Endaurel Hospital, New York City; Fellow in Research, Harvard Medical School; formerly Assistant in Applied Anatomy and Pathology in Otolaryngology, Washington University Medical School, St. Louis, Mo. The Williams and Wilkins Company, Baltimore, 1947. Price \$18.00. Cloth with 1053 pages.
- Leprosy.** By *Sir Leonard Rogers*, K.C.S.I., C.I.E., M.D., F.R.C.P., F.R.C.S., F.R.S., I.M.S. (Retired); Late Physician and Lecturer, London School of Tropical Medicine; Professor of Pathology, Calcutta Medical College; and *Ernest Muir*, C.I.E., M.D., F.R.C.S. (Edin.); Medical Secretary, British Empire Leprosy Relief Association; Late Research Worker in Leprosy at the School of Tropical Medicine and Hygiene, Calcutta. The Williams and Wilkins Company, Baltimore, 1946. Price \$7.00. Cloth with 266 pages.
- The Diagnosis and Treatment of Bronchial Asthma.** By *L. N. Gay*, Ph.D., M.D.; Assistant Professor of Medicine of the Johns Hopkins University School of Medicine; Director of the Allergy Clinic of the Johns Hopkins Hospital; with a foreword by *W. R. Longcope*, A.B., M.D.; Professor of Medicine of the Johns Hopkins University School of Medicine; Physician-in-Chief of the Johns Hopkins Hospital. The Williams and Wilkins Company, Baltimore, 1946. Price \$5.00. Cloth with 324 pages.
- The Pathology of Traumatic Injury.** By *James V. Wilson*, M.D., M.R.C.P. (Lond.); Major R.A.M.C. (T.); Pathologist to Harrogate and District General Hospital and the Royal Bath Hospital, Harrogate; sometime Associate Professor of Pathology, Farouk I. University, Alexandria; Pathologist to the Anglo-Swiss Hospital, Alexandria; D.A.D.P., Malta Command, 1940-1943. Foreword by *P. H. Mitchiner*, C.B., C.B.E., T.D., M.D., M.S., F.R.C.S., D.Ch., K.H.S.; Major-General A.M.S.; Surgeon St. Thomas' Hospital. The Williams and Wilkins Company. Baltimore, 1946. Price \$6.00. Cloth with 182 pages.
- Concise Anatomy.** By *L. F. Edwards*, Ph.D.; Professor of Anatomy, the Ohio State University, Columbus, Ohio. The Blakiston Company, Philadelphia, 1947. Cloth with 514 pages and 324 illustrations.
- Paravertebral Block in Diagnosis, Prognosis, and Therapy.** By *Felix Mandl*, M.D., F.I.C.S.; Professor of Surgery, Hadassah University Hospital, Jerusalem. Translated by *Gertrude Kallner*, M.D. Grune and Stratton, New York, 1947. Price \$6.50. Cloth with 319 pages.



- A History of the American Medical Association 1817 to 1917.** By *Morris Fishbein*, M.D. W. B. Saunders Company, Philadelphia, 1917. Cloth with 1185 pages.
- Diseases of Metabolism. A Text for the Practitioner.** By *G. G. Duncan*, M.D.; Director of Medical Division, Pennsylvania Hospital; Clinical Professor of Medicine, Jefferson Medical College, Philadelphia, Pa. W. B. Saunders Company, Philadelphia, 1947. Cloth with 995 pages.
- Cancer—Diagnosis, Treatment, and Prognosis.** by *L. V. Ickerman*, M.D.; Pathologist to the Ellis Fischel State Cancer Hospital; Assistant Professor of Pathology, Washington University School of Medicine, St. Louis; and *Juan A. del Regato*, M.D.; Radiotherapist to the Ellis Fischel State Cancer Hospital; formerly Assistant to the Radium Institute of the University of Paris. The C. V. Mosby Company, St. Louis, 1947. Price \$20.00. Cloth with 1058 pages, 745 text illustrations and 42 color reproductions.
- Recent Advances in Clinical Pathology.** General Editor, *S. C. Dyke*, D.M. (Oxon.), F.R.C.P. (Lond.); Section Editors: Bacteriology, *R. Cruickshank*, M.D. (Aberd.), F.R.C.P. (Lond.); Biochemistry, *E. N. Allott*, B.Sc., B.M. B. Ch. (Oxon.), F.R.C.P. (Lond.); Hematology and Cytology, *B. L. Della Vida*, M.D. (Rome); Histology, *A. H. T. Robb-Smith*, M.D. (Lond.). The Blakiston Company, Philadelphia, 1947. Cloth with 462 pages, 34 plates, and 19 text figures.
- An Introduction to Bacteriological Chemistry.** By *C. G. Anderson*, Ph.D. (Birm.); Dip. Bact. (Lond.); formerly Lewis Cameron Teaching Fellow, Bacteriology Department, University of Edinburgh. The Williams & Wilkins Company, Baltimore, 1946. Price \$5.00. Cloth with 469 pages.
- Pulmonary Tuberculosis. A Handbook for Students and Practitioners.** By *R. Y. Keers*, M.D., M.R.C.P. (Edin.), F.R.F.P.S. (Glas.); Medical Director, Red Cross Sanatoria of Scotland; Late Assistant Medical Superintendent, British Sanatorium, Montana, Switzerland; First Assistant Medical Officer, King Edward VII Sanatorium, Midhurst; and *B. G. Rigden*, M.R.C.S. (Eng.), L.R.C.P. (Lond.); First Assistant Medical Officer, Red Cross Sanatoria of Scotland; Late Resident Medical Officer, East Anglian Sanatorium, Mayland; Resident Medical Officer, Kelling Sanatorium, Holt. The Williams & Wilkins Company, Baltimore, 1946. Cloth with 193 pages, 124 glossy photographs.
- Medical Aspects of Growing Old.** By *A. T. Todd*, M.D. (Edin.), M.R.C.P. (Lond.); Honorary Physician, Bristol Royal Infirmary. John Wright and Sons, Ltd., Bristol, 1946. Cloth with 161 pages.
- Physicians Desk Reference to Pharmaceutical Specialties and Biologicals (in five sections).** *J. Morgan Jones*, Editor and Publishers. Published by Medical Economics, Inc., Rutherford, N. J., 1946. Cloth with 380 pages.
- The Causation of Appendicitis.** By *A. Bendle Short*, M.D., B.S., B.Sc., F.R.C.S.; Professor of Surgery, University of Bristol; Surgeon, Bristol Royal Infirmary. The Williams & Wilkins Company, Baltimore, 1946. Price \$2.50. Cloth with 77 pages.
- An Approach to Social Medicine.** By *John D. Kershaw*, M.D. (Lond.), D.P.H.; Medical Officer of Health, Accrington. The Williams & Wilkins Company, Baltimore, 1946. Price \$4.50. Cloth with 305 pages.
- Textbook of Medicine.** By various authors; Edited by *Sir John Conybeare*, K.B.E., M.D., D.M. (Oxon.), F.R.C.P.; Physician to Guy's Hospital, London. The Williams & Wilkins Company, Baltimore, 1946. Cloth with 1151 pages, 25 illustrations and 31 x-ray plates.

**Medical Disorders of the Locomotor System.** By *Ernest Fletcher*, M.A., M.D. (Cantab.), M.R.C.P.; Physician to the Arthritis Clinic and Lecturer on the Rheumatic Diseases, Royal Free Hospital; Physician, Queen Mary's Hospital for the East End and the British Red Cross Clinic for Rheumatism; Consulting Physician to the British Legion; Heberden Medallist and Lecturer in Rheumatism; Member of the Research and Scientific Advisory Committees, Empire Rheumatism Council; Late Physician-in-Charge of a Medical Division, Emergency Medical Service, Ministry of Health; Consulting Physician to the Kent County Council. The Williams & Wilkins Company, Baltimore, 1947. Price \$11.00. Cloth with 616 pages.

**Forensic Medicine.** By *Keith Simpson*, M.D. (Lond.); Lecturer in Forensic Medicine to Guy's Hospital, London; Examiner in Forensic Medicine, University of London. The Williams & Wilkins Company, Baltimore, 1947. Price \$4.50. Cloth with 322 pages.

**Atlas of Histopathology of the Skin.** By *G. H. Percival*, M.D., Ph.D., F.R.C.P.E., D.P.H.; Grant Professor of Dermatology, University of Edinburgh; Physician to the Skin Department, Royal Infirmary, Edinburgh; Consultant Dermatologist to Edinburgh Municipal Hospitals; *A. Murray Drennan*, M.D., F.R.C.P.E., F.R.S.E.; Professor of Pathology, University of Edinburgh; Honorary Pathologist to the Royal Infirmary, Edinburgh; Director of Pathological Services to the Municipal Hospitals, Edinburgh; Director of Scottish Mental Hospitals Pathological Scheme; and *T. C. Dodds*, F.I.M.L.T., F.I.B.P., F.R.P.S.; Laboratory Supervisor, Department of Pathology, University of Edinburgh; Lecturer to the Society of Radiographers (Fellowship Course) Scottish Branch. The Williams & Wilkins Company, Baltimore, 1947. Price \$16.00. Cloth with 419 pages and 370 colored plates.

**A Handbook of Diseases of Children.** By *Bruce Williamson*, M.D. (Edin.), F.R.C.P. (Lond.); Physician, Children's Department, Royal Northern Hospital, London; Physician, Prince of Wales General Hospital, London; Consulting Physician, Wellhouse Hospital, Barnet; Consulting Physician, Enfield War Memorial Hospital; Consulting Physician, Brentwood and District Hospital; Late Examiner in Medicine, General Nursing Council; Consulting Physician, Pioneer Health Centre, Peckham; etc. The Williams & Wilkins Company, Baltimore, 1947. Price \$4.50. Cloth with 390 pages and 86 illustrations.

**Color Atlas of Hematology.** By *Roy E. Kracke*, M.D.; Dean and Professor of Clinical Medicine, Medical College of Alabama, Birmingham, Ala. J. B. Lippincott Company, Philadelphia, 1947. Cloth with 168 pages and 34 plates.

**Dermatologic Clues to Internal Disease.** By *H. T. Behrman*, M.D.; Assistant Clinical Professor of Dermatology, New York University College of Medicine; Adjunct Dermatologist, Mount Sinai Hospital and Beth Israel Hospital; Associate Dermatologist, Hillside Hospital; Diplomate of the American Board of Dermatology and Syphilology; Fellow of the American Academy of Dermatology and Syphilology. Grune and Stratton, Inc., New York, 1947. Price \$5.00. Cloth with 165 pages.

**Surgical Pathology.** By *William Boyd*, M.D., Dip. Psychiat. M.R.C.P. (Edm.), F.R.C.P. (Lond.), LL.D. (Sask.), M.D. (Oslo), F.R.S.C.; Professor of Pathology, The University of Toronto. W. B. Saunders Company, Philadelphia, 1947. Cloth with 817 pages and 530 illustrations, including 22 color figures.

**The 1946 Year Book of Endocrinology, Metabolism and Nutrition.** Endocrinology: edited by *Willard O. Thompson*, M.D.; Clinical Professor of Medicine, University of Illinois College of Medicine; Attending Physician (Senior Staff), Henrotin Hospital; Attending Physician, Grant Hospital of Chicago. Metabolism and Nutrition: edited by *Tom D. Spies*, M.D.; Associate Professor of Medicine, University of Cincinnati School of Medicine; Director, Nutrition Clinic, Hillman Hospital, Birmingham, Ala. The Year Book Publishers, Inc., Chicago, 1947. Price \$3.75. Cloth with 551 pages.

- The Acute Infectious Fevers.** By *Alexander Jock*, D.S.C., M.D. (Ed.), F.R.C.P. (Ed.), D.P.H., D.T.M. and H.: Medical Superintendent, City Hospital, Edinburgh; Lecturer on Infectious Disease to the University of Edinburgh; formerly Medical Superintendent, North-Western Hospital, London.
- The Pharmacopoeia of the United States of America (The United States Pharmacopoeia)** by authority of the United States Pharmacopoeial Convention meeting at Washington, D. C., May 14 and 15, 1940. Prepared by the Committee of Revision and Published by the Board of Trustees. Officials from April 1, 1947. Mack Printing Company, Easton, Pa., 1947. Cloth with 923 pages.
- May's Manual of the Diseases of the Eye.** Revised and edited by *Charles A. Perera*, M.D.; Assistant Clinical Professor, College of Physicians and Surgeons, Columbia University, New York; Assistant Attending Ophthalmologist, Presbyterian Hospital, New York. The Williams & Wilkins Company, Baltimore, 1947. Price \$4.00. Cloth with 500 pages, 387 illustrations, including 32 plates, and 93 colored figures.
- Office Immunology Including Allergy.** By *Marion B. Sulzberger*, M.D.; Professor of Clinical Dermatology and Syphilology and Director, New York Skin and Cancer Unit, New York Post-Graduate Medical School and Hospital; *W. C. Spain*, M.D.; Clinical Professor of Medicine, New York Post-Graduate Medical School and Hospital; *Rudolf L. Baer*, M.D.; Instructor in Dermatology and Syphilology, New York Skin and Cancer Unit, New York Post-Graduate Medical School and Hospital; *Abram Kanof*, M.D.; Adjunct Pediatrician, Jewish Hospital, Brooklyn; *Alfred J. Weil*, M.D.; Lederle Laboratories Division, American Cyanamid Company; and *Naomi M. Kanof*, M.D.; Associate Attending in Dermatology, Garfield Memorial and Children's Hospitals, Washington, D. C. Edited by *M. B. Sulzberger* and *Rudolf L. Baer*. Price \$6.50. The Year Book Publishers, Inc., Chicago, 1947. Cloth with 408 pages.
- The Christchurch Hospital Medical Manual.** Edited by *C. T. Hand Newton*, D.S.O., M.D., F.R.A.C.P., F.R.C.S. (Edin.). N. M. Peryer Ltd., Christchurch, New Zealand, 1947. Cloth with 106 pages.
- Recent Advances in Endocrinology.** By *A. T. Cameron*, C.M.G., M.A., D.Sc. (Edin.), F.R.I.C., F.R.S.C.; Professor of Biochemistry, Faculty of Medicine, University of Manitoba; Biochemist, Winnipeg General Hospital. The Blakiston Company, Philadelphia, 1947. Cloth with 434 pages and 74 figures.
- Gynecology With a Section on Female Urology.** By *Lawrence R. Wharton*, Ph.B., M.D.; Assistant Professor of Gynecology, the Johns Hopkins Medical School; Assistant Attending Gynecologist, the Johns Hopkins Hospital; Consultant in Gynecology, the Union Memorial Hospital, Hospital for the Women of Maryland, Sinai Hospital and Church Home and Infirmary. W. B. Saunders Company, Philadelphia, 1947. Cloth with 1000 pages and 479 illustrations.
- Nutritional Disorders of the Nervous System.** By *John D. Spillane*, B.Sc., M.D. (Wales), M.R.C.P. (Lond.); Assistant Physician and Neurologist, Cardiff Royal Infirmary; formerly Adviser in Neurology to the Middle East Forces; Commonwealth Fund Fellow at the Neurological Institute of New York City and at the Massachusetts General Hospital, Boston. The Williams & Wilkins Company, Baltimore, 1947. Price \$5.00. Cloth with 267 pages.
- Symptoms and Signs in Clinical Medicine. An Introduction to Medical Diagnosis.** By *E. Noble Chamberlain*, M.D., M.Sc., F.R.C.P.; Lecturer in Medicine, University of Liverpool; Physician to Out-patients, Royal Liverpool United Hospital, Royal Infirmary Branch; Visiting Physician, Smithdown Road Hospital, Liverpool; Formerly Beit Memorial Research Fellow. The Williams & Wilkins Company, Baltimore, 1947. Price \$8.00. Cloth with 440 pages.

- Dying, Apparent-Death and Resuscitation.** By *S. Jellinek*, M.D.; Professor of Electropathology at the University of Vienna, Lauréat de l'Institut de France. The Williams & Wilkins Company, Baltimore, 1947. Price \$3.50. Cloth with 216 pages.
- Pharmaco-Therapeutic Notebook.** By *H. W. Tomski*; Member of the Pharmaceutical Society; Diploma in Biochemical Analysis. The Williams & Wilkins Company, Baltimore, 1947. Price \$1.50. Cloth with 259 pages.
- Internal Medicine in General Practice.** By *Robert Pratt McCombs*, B.S., M.D., F.A.C.P.; Assistant Professor of Medicine and Director of Postgraduate Teaching, Tufts College Medical School; Senior Attending Physician, the Joseph H. Pratt Diagnostic Hospital; Diplomate of the American Board of Internal Medicine. W. B. Saunders Company, Philadelphia, 1947. Cloth with 697 pages.
- The American Illustrated Medical Dictionary.** A complete dictionary of the terms used in medicine, surgery, dentistry, pharmacy, chemistry, nursing, veterinary science, biology, medical biography, etc., with the pronunciation, derivation, and definition by *W. J. Newman Dorland*, A.M., M.D., F.A.C.S.; Lieut.-Colonel, M.R.C., U. S. Army; Member of the Committee on Nomenclature and Classification of Diseases of the American Medical Association; Editor of *American Pocket Medical Dictionary*. Twenty-first edition. W. B. Saunders Company, Philadelphia, 1947. Cloth with 1660 pages, 880 illustrations.
- An Introduction to Biochemistry.** By *William Robert Fearon*, M.A., Sc.D., M.B.; Fellow of Trinity College, and Professor of Biochemistry, University of Dublin; Fellow of the Royal Institute of Chemistry; Member of the Royal Irish Academy. Grune & Stratton, New York, Inc., 1947. Price \$6.00. Cloth with 541 pages.

## EXPERIMENTAL CHEMOTHERAPY OF FILARIASIS

### I. EXPERIMENTAL METHODS FOR TESTING DRUGS AGAINST NATURALLY ACQUIRED FILARIAL INFECTIONS IN COTTON RATS AND DOGS

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THE exposure of our armed personnel to filariasis in endemic regions of the Pacific theater of war stimulated the search for new filaricidal drugs in this country. In 1945 a report on the known methods of treatment for filariasis was prepared by Temkin for the Division of Medical Sciences of the National Research Council.<sup>1</sup> In this report are listed the results obtained by various investigators, chiefly with derivatives of heavy metals, in their attempts to cure the disease in man.

Recent reports by Brown<sup>2</sup> and by Culbertson, Rose, and Oliver-Gonzalez<sup>3-5</sup> demonstrate promising results in man by the use of Lithium Antimony Thiomalate and Neostibosan.

Until very recently the search for new drugs to treat filariasis has been limited because of the lack of a small, easily available laboratory host. A large proportion of the early experimental chemotherapy on this disease was carried out in dogs infected with *Dirofilaria immitis*.<sup>6</sup> The routine screening of new compounds in the dog, however, is virtually impossible because of the relatively large amounts of drug needed to treat a single animal. Various investigators have reported the successful treatment of *Dirofilaria* infections with antimonials, and Fuadin<sup>7, 8</sup> is at present the drug of choice for the treatment of heartworm infections in dogs. Otto and Maren<sup>9</sup> have reported the use of a new arsenical for treating this infection.

*Litomosoides carinii*, a filarial parasite of the cotton rat (*Sigmodon hispidus*), is now being used in many laboratories for the bio-assay of filaricidal drugs.<sup>10-13</sup> The vector of this parasite has been found to be the tropical rat mite (*Liponyssus bacoti*)<sup>14, 15</sup> and several papers have been published regarding the host-parasite relationships.<sup>16-19</sup> Some information has been made available regarding the effects of chemical compounds other than antimonials against this infection,<sup>10, 11, 23</sup> chiefly the cyanine dyes.

Since April, 1945, filaria-infected cotton rats have been used in these laboratories for the routine screening of various nonmetallic, organic compounds for filaricidal properties. The methods used for bio-assay, both in cotton rats and dogs, and the results of tests on compounds in cotton rats are reported herein.

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## EXPERIMENTAL METHODS IN COTTON RATS

Naturally infected cotton rats<sup>o</sup> were used as experimental hosts. Animals of various ages and both sexes were used. The number of microfilariae present in the blood stream upon arrival varied considerably, and no method was devised for determining the number of adult worms present in the pleural cavity before treatment was initiated.

Bell and Brown<sup>18</sup> have demonstrated that the microfilariae of *Litomosoides carinii* do not exhibit nocturnal or diurnal periodicity, and our studies confirm these observations (Table II).

*Method of Counting Microfilariae.*—The first criterion which was used for determining the effect of a new drug against this infection was the behavior of the microfilarial count in the peripheral blood. Rather than strive for absolute, quantitative values it was apparent that much time would be saved by developing a quick qualitative method for examining the blood. Thin smears were made from one drop of blood obtained from the cut tails or toes of the rats. These were air dried and stained with Giemsa's stain, with or without dehemoglobinization. The presence of intact red cells did not materially detract from the ease with which embryos could be found under a low-power objective of the microscope. Generally, from 25 to 100 microscopic fields were counted, but where very few microfilariae were present, 100 fields were always counted. The count from all animals was then expressed per 100 microscopic fields.

It is recognized that this method is subject to considerable error, but our interest was primarily in an all-or-none effect, and all animals under treatment were handled in the same way.

In our first experimental series, microfilarial counts were made on the day before treatment was initiated and every day thereafter for from two to four weeks. In later series this was considered unnecessary, and counts were made only before treatment and on the day of autopsy.

*Microfilarial Counts in Untreated Rats.*—In Tables I, II, and III data are given from untreated rats for varying periods of observation. In each case it will be noted that considerable fluctuation occurred in microfilarial counts, regardless of how often slides were examined.

The weekly records (Table III) provide the most pertinent information. It will be noted that all of the rats showed a marked increase in microfilariae over a period of several weeks observation. This denotes that the animals were probably infected with worms which had not all reached maturity when received and subsequently discharged increasingly larger numbers of embryos. Similar observations have been made in human infections in children.<sup>3</sup> The significance of this observation is that decreases in microfilariae which occur in treated animals are probably greater than actually observed over a period of time.

In only one untreated rat of sixty-five which have been studied has the microfilarial count dropped spontaneously to zero.

*Description of Adult Worms in Cotton Rats.*—*Litomosoides carinii* is a parasite of the pleural cavity in cotton rats and with few exceptions in more

<sup>o</sup>Obtained from the Hegener Research Supply Company, Sarasota, Fla.

TABLE I. UNTREATED COTTON RATS—MICROFILARIAL COUNTS ON ALTERNATE DAYS

RAT	DAY																	
	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35
41	244	156	240	112	316	675	248	308	268	180	336	128	396	152	368	161	161	196
108	252	372	144	360	248	120	61	56	201	30	252	312	428	101	88	168	80	220
125	204	336	212	244	576	244	192	328	268	124	296	22	308	212	260	221	272	452
182	272	328	236	204	188	184	120	192	184	576	301	272	532	344	412	610		
200	34	56	64	32	34	30	44	24	24	44	52	60	96	56	7	46		
193	61	44	40	48	12	32	56	24	28	52	52	61	8	68	32	121		
195	116	60	108	112	108	104	64	60	68	196	61	44	140	181	72	214		
198	176	88	372	120	140	260	212	92	360	160	160	268	324	472	268	244		

TABLE II. UNTREATED COTTON RATS—MICROFILARIAL COUNTS EVERY TWO HOURS

MICROFILARIAE PER 100 LOW-POWER FIELDS																										
PAT.	HOUR																									
	0	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48	
227	32	14	30	18	6	10	16	12	14	16	14	14	44	44	20	60	30	56	88	76	56	60	40	108	44	
241	68	32	36	4	44	40	104	96	88	44	18	48	28	26	128	100	81	172	181	144	180	152	116	76	96	
247	60	24	16	96	18	10	56	62	42	8	32	40	28	16	76	51	40	56	60	76	26	*				
249	16	18	16	4	12	0	20	4	16	20	24	10	6	8	16	20	14	12	30	30	20	40	20	60	8	

**•, Death.**

TABLE III. UNTREATED COTTON RATS—WEEKLY MICROFILARIAH COUNTS

MICROFILARIAE PER 100 LOW-POWER FIELDS																		
RAT	WEEK																	
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
150	75	88	176	420	220	540	420	332	220	212	1,152	392	792	201	236	*		
151	34	22	56	160	232	460	216	400	920	552	596	536	668	288	480	618	1,004	1,070
153	56	168	276	332	398	700	332	948	*									
154	42	84	44	132	76	260	260	356	296	380	536	940	-	-	508	520	596	440
155	40	24	124	128	108	312	160	212	*									
156	116	88	300	396	264	580	176	604	*									
157	32	60	152	240	432	352	372	496	*									
158	40	148	26	68	40	372	268	308	*									
41	244	112	308	400	152	164	140	184	168	132	236	228	261	116	236	332		
108	252	360	56	142	104	564	112	152	312	200	76	92	176	248	140	312		
125	204	244	328	188	212	388	344	352	632	452	248	216	480	388	380	361		
182	200	204	120	304	344	400	652	364	816	500	528	652						
200	44	32	44	52	132	92	56	116	120	80	80							
193	56	48	24	52	68	-	44											
195	72	112	60	64	184	-	136	416	384	140	120	368	400					
198	212	120	92	160	472	-	600	284	632	236	592	520	1480					
201	20	22	18	8	0	-	*											
227	32	18	16	-	104	64	44	72	76	56								
241	68	16	84	-	208	100	144	200	161	168								
362	40	12	22	-	72	60	28	44	34	48								
368	40	56	68	14														
388	40	56	20	12														
388	560	410	240	720														
389	120	780	430	88														

\*, No reading taken.

\*, Death.



TABLE IV. UNTREATED COTTON RATS—PER CENT DIFFERENCE FROM ORIGINAL MICROFILARIAL COUNT AT END OF OBSERVATION PERIOD

RAT	NUMBER WEEKS OBSERVED	PER CENT DIFFERENCE FROM ORIGINAL COUNT	
		INCREASE	DECREASE
150	14	251	
151	17	3,017	
153	7	1,593	
154	17	917	
155	7	430	
156	7	420	
157	7	1,150	
158	7	670	
41	15	36	
108	15	23	
125	15	78	
182	11	216	
200	11	81	
193	6		22
195	12	455	
198	12	598	
201	4		100
227	9	75	
241	9	147	
249	9	200	
362	3		65
368	3		70
388	3	28	
389	3		27

than 1,000 autopsies adult worms have never been found in any other site. In a few cases no worms were found in the pleural cavity, but several occurred free in the abdominal cavity. When many worms were present they occurred intertwined in masses, generally applied to the dorsal wall, or were located in the pockets formed between the lobes of the lungs and the diaphragm. Frequently, they were applied to the pericardial membrane or to the outer surface of the lungs. Macroscopic host tissue reactions to the presence of living worms rarely were found, and when they were removed the pleural walls appeared shiny, glistening, and apparently undamaged.

Living worms recovered from the pleural cavity invariably undergo writhing movement when placed in 0.8 per cent saline solution and continue this activity for several hours at room temperature. They are slick, shiny, and transparent when viewed under a binocular dissecting microscope, and the internal structure is clearly defined.

In some animals in which dead worms were found at autopsy, particularly those in which large numbers of worms were killed, extensive adhesions of the heart and lungs to the pleural cavity wall frequently occurred. Isolated dead worms showed no movement whatever when placed in 0.8 per cent saline and were stretched out for their full length. When viewed under a microscope they had a dull, opaque appearance, and the internal structure was not clearly defined.

When dead worms occurred in masses they were sometimes, but not always, encased in a caseous, purulent "exudate." Culbertson and Rose<sup>12</sup> have described a similar substance surrounding dead worms following treatment with antimony derivatives. Bertram, Unsworth, and Gordon<sup>16</sup> have also described this substance surrounding dead worms in white albino rats which were

experimentally infected with *Litomosoides carinii* through the use of the vector *Liponyssus bacoti*. We have found it to consist largely of leucocytes and fat and believe that it represents a response of the host to the presence of decaying worms.

In later stages of decay, dead worms occurred in tight, shrunken masses, were yellowish in color, and were fragmented.

Of sixty-five untreated rats which were subjected to autopsy, nine showed a small number of dead worms. In no case, however, were large numbers of dead worms present, and caseous or purulent material was rarely found.

*Methods of Dosage and Evaluation of Drugs in Cotton Rats.*—In common with all host-parasite relationships used for evaluating the effectiveness of drugs, several different methods are available to the investigator for determining whether new chemical compounds are active against filariasis in cotton rats. The amount of drug given, route of administration, number of days of treatment, frequency of treatment, and time of autopsy are all variables to which some degree of standardization must be assigned if comparisons are to be made with known filaricidal substances.

Using cotton rats as experimental hosts, the only published reports in detail relative to the effectiveness of treatment with antimony compounds are those of Culbertson and Rose,<sup>12</sup> Culbertson and Price,<sup>13</sup> and Lawton and co-workers.<sup>20</sup> The effect of cyanine dyes against filariasis in cotton rats has been presented recently.<sup>10, 11, 23</sup> Culbertson and Rose demonstrated that large intramuscular doses of Neostam and Neostibosan (400 to 800 mg. per kilogram) administered once daily, with varying intervals between doses, killed the adult worms and produced a gradual disappearance of microfilariae from the peripheral blood. Essentially the same results were obtained by Culbertson and Price<sup>13</sup> with Stibanose (Solustibosan). We have been able to confirm these results in a small series of cotton rats.

Due to the paucity of information regarding the effectiveness of compounds other than antimonials against filariasis in any vertebrate host, we believed it important to study effects produced by new compounds against both microfilariae and macrofilariae in our screening tests.

In our program, compounds received for testing were administered orally or intraperitoneally to cotton rats, depending upon their solubility. If a sufficient quantity of the drug was available, 100 to 200 mg. per kilogram were given orally, or 25 to 50 mg. per kilogram intraperitoneally, as a starting dose. Previous experience with drugs used for screening purposes in other host-parasite relationships frequently determined the starting dose. If the animals survived, treatment was administered twice daily for from two to four weeks. For preliminary screening, from one to three animals were used for each test. If the compound proved toxic, and a sufficient supply was available, the dose was halved and retested. Active compounds were tested at many dose ranges.

Insoluble drugs were suspended in a 2.5 per cent solution of corn starch and were administered orally through a 2½ inch, 18-gauge hypodermic needle with a blunted end. Soluble drugs were dissolved in water and were administered intraperitoneally through a ½ inch, 23-gauge hypodermic needle.

In general, autopsies were performed at intervals of from two to four weeks after cessation of treatment, although this procedure was not strictly adhered to in the case of compounds showing suggestive activity in previous tests. Large groups of animals were used when compounds showed an effect against either microfilariae or macrofilariae, and autopsies were performed at various intervals after cessation of treatment. In cases where large masses of living or dead worms were present no attempt was made to count the exact number.

When precipitous and sustained reductions in microfilariae occurred during treatment, or dead adult worms were found at autopsy following treatment, repeated tests were made on compounds producing these effects. Animals with low initial microfilaria counts (namely, less than 10 per 100 fields) were found to be very unsatisfactory for assay purposes, since reductions in microfilariae which might occur were well within the limits of nontreated controls.

Certain obvious sources of error are apparent in the procedures described for the routine screening of compounds against this infection. Longer periods of treatment, more frequent treatment, or longer intervals between cessation of treatment and autopsy might have revealed more active compounds than have as yet been found. Since practically nothing was known about the absorption, excretion, or toxicity of the compounds before tests were made, however, it became necessary to adopt some uniform plan and to treat all compounds alike until experience demonstrated that other methods would produce better results. Similar situations have been experienced in all screening programs, using various host-parasite relationships. Very little is known concerning the mode of action of known filaricidal drugs, and it seemed to us that the trial-and-error method of bio-assay in searching for new drugs was the best method of approach.

#### EXPERIMENTAL METHODS IN DOGS

Adult *Dirofilaria immitis* commonly occur in the right ventricle of the heart or may be found in the pulmonary artery near the heart. The number of adult worms probably determines the severity of symptoms; namely, emaciation, weakness, jaundice, and a persistent cough. Worms which may be killed by drugs, and probably some which die naturally, are found in the terminal branches of the pulmonary artery within the lung. This has been described by Wright and Underwood<sup>7</sup> in dogs treated with Fuadin.

If a large number of worms are present in the heart when treatment with an active compound is begun, it has been assumed, and with some good substantiating evidence, that worms which are killed quickly and simultaneously will produce an embolus in the pulmonary artery and kill the dog. Heavily infected dogs, namely, those which show a high microfilarial count with severe symptoms, are therefore considered poor risks for treatment.

In our experiments dogs have been used only for determining the effectiveness of compounds which showed activity in the cotton rat. Essentially the same procedures for treatment were used as described for the cotton rat, and previous results from the rats provided a rough estimate of the probable tolerated dosage in dogs. Oral doses were administered in capsules.

Microfilarial counts from dogs were made by withdrawing 0.05 c.c. of blood from the large vein in one of the forelegs, placing a 22 by 40 mm. cover slip over the blood on a glass slide, and counting the entire fresh preparation. Quantitative methods were more feasible in the dogs than in cotton rats, since fewer animals were used, and measured samples of blood could be withdrawn frequently without seriously disturbing the host.

In some untreated dogs, as well as treated dogs with initial low embryo counts (from 2 to 10 microfilariae per 0.05 c.c.), no adult worms were found at autopsy. This has been experienced by other investigators.

As has been mentioned for cotton rats, dogs with low initial microfilarial counts are unsatisfactory for assay purposes. Any decrease which might occur in the microfilarial count in such animals following treatment cannot be measured either qualitatively or quantitatively. Moreover, a large percentage of these cases invariably reveal no adult worms at autopsy, and the results of treatment are therefore exceedingly questionable.

Following treatment with Puadin, the microfilarial count is reduced within one to several weeks and varying numbers of dead worms are found in the lungs at autopsy.<sup>7, 8</sup> It is frequently necessary to push the dosage to the limit of tolerance, however, and in some cases death occurs from acute toxicity.

For determining the effectiveness of new compounds against *Dirofilaria immitis* infections in dogs, we have used three criteria: (1) a rapid and sustained reduction in microfilariae, (2) presence of dead worms in the terminal blood vessels within the lung at autopsy, and (3) improved physical condition of the animal. The improvement in the physical conditions of animals following treatment is not a certain sign that cure has been effected if it is not accompanied by the destruction of the worms. We have frequently received dogs for treatment which were emaciated, sickly, and unkempt. Some of these animals were caged and fed adequately for a number of weeks preceding treatment, were freed of fleas, and were cured of mange, and all improved in appearance markedly during their pretreatment period, in spite of the fact that a relatively large number of microfilariae were present in the peripheral blood.

#### COMPOUNDS TESTED FOR FILARICIDAL ACTIVITY IN COTTON RATS

In our screening program, 517 organic, nonmetallic compounds have been tested for activity against *Litomosoides carinii* in the cotton rat. These included 78 organic dyes (phenazines, thiazines, oxazines, triphenylmethanes, phenoxazines, acridines, anthraquinones, and fluorenes); 31 acids and derivatives, 21 aromatic amines, 13 aromatic ketones, 7 amino Alkanols, 8 methanes; 16 oxazolidines, 62 guanidines, melamines, biguanides, and guanyles, 112 piperazines, 15 pyridines, 8 metanilamides, 20 sulfonamides, 9 ureas, thioureas, and urethrones, 22 sulfides, sulfones, and sulfonic acids, 16 pyrimidines and pyrazines, 22 piperidines, 4 triazines, 5 quinolines and isoquinolines, 7 cyanamides and dicyandiamides, 6 imidazoles and pyrazoles, 8 dioxanes and morpholines, and 25 miscellaneous compounds, including antibiotics and plant extracts.

Several types of compounds showed activity in the cotton rat, either against microfilariae or macrofilariae or both. The best balance between activity and

toxicity, however, was found in the piperazines. The first piperazine tested which showed filaricidal activity was 1-carbethoxy-4-methylpiperazine hydrochloride (180-C). By the oral or intraperitoneal administration of doses ranging from 12.5 to 400 mg. per kilogram, this compound produced a rapid reduction of microfilariae in the peripheral blood and showed suggestive activity against the adult worms. Because of the unique effects produced by this compound and related piperazines, detailed results are presented in separate papers.<sup>21, 22</sup>

Data concerning active compounds other than piperazines will be presented when more complete information has been obtained.

#### DISCUSSION

The evaluation of new chemical compounds for therapeutic effectiveness against any naturally acquired infection in animals is subject to considerably more error in the interpretation of results than is the case where standardized infections are produced by introducing known numbers of parasites into laboratory hosts. In the case of such highly standardized infections, for example, as *Plasmodium lophurae* in the duck and *Trypanosoma equiperdum* in mice, a single screening test will often give definite information regarding the effect of a new compound. Moreover, in both of these laboratory infections, several known compounds of widely different chemical structure can be used as control drugs.

For testing new compounds for filaricidal properties, no standardized laboratory infection has as yet been devised. The vectors of both *Litomosoides carinii* in the cotton rat<sup>14, 15</sup> and *Dirofilaria immitis* in the dog are known, and it is quite probable that in the future a method will be available for testing drugs against experimental infections of known character in these animals. The use of *Dirofilaria immitis* in the dog, however, will probably never prove satisfactory for a large scale screening programs, due to the obvious disadvantages of a large experimental host.

In evaluating the effects of the different types compounds tested in these laboratories, an attempt was made in each case to determine the effect produced upon both microfilariae and macrofilariae in the cotton rat. Sometimes this was not possible, since the animals died while under treatment and the supply of drug was not sufficient to make a second trial. In such cases, when no reduction in microfilariae occurred during treatment and the animals died before the time planned for autopsy, the drug was listed as inactive. Obviously, this cannot be considered a true appraisal of the drug in every case, since compounds are known which produce no direct effect upon the microfilariae but do kill the adult worms.<sup>12, 13</sup>

The time which elapses between cessation of treatment and autopsy is also a factor which may influence the evaluation of new compounds, as will be demonstrated in subsequent papers.<sup>21, 22</sup>

Nontreated control animals and animals treated with filaricidal antimony compounds were not included in each protocol in our screening program. The effects of antimony derivatives against *Litomosoides carinii* have been substan-

tially demonstrated<sup>12, 13</sup> and the course of untreated infections was studied at various times. Since the animals received varied in age, weight, and duration and intensity of infection, and because there was no way for determining how many adult worms were present, any suggestion of activity produced by new compounds was followed by repeated tests.

Recognizing the variables which accompany the use of naturally infected cotton rats for screening new compounds against filariasis, we believe that this host-parasite relationship provides a useful tool in the search for new filaricides, particularly since no other small host is available which will serve the purpose. The correlation between activity in cotton rats, dogs, and human beings infected with filaria has yet to be determined.

#### SUMMARY

Methods used in testing new chemical compounds for filaricidal activity in naturally infected cotton rats and dogs are described. Data are given relative to untreated infections in cotton rats, and the types of compounds tested are mentioned. The filaricidal effect of 1-carbethoxy-4-methylpiperazine hydrochloride is discussed.

#### REFERENCES

1. Temkin, O.: A Report on the Medicinal Treatment of Filariasis Bancrofti, Office of Medical Information, Division of Medical Sciences, National Research Council, Washington, 1945, 69 pp.
2. Brown, H. W.: The Treatment of Filariasis (*Wuchereria Bancrofti*) With Lithium Antimony Thiomaleate, *J. A. M. A.* 125: 952-958, 1944.
3. Culbertson, J., Rose, H., and Oliver-Gonzalez, J.: Chemotherapy of Human Filariasis by the Administration of Neostibosan, *Am. J. Trop. Med.* 25: 271-274, 1945.
4. Culbertson, J., Rose, H., and Oliver-Gonzalez, J.: The Chemotherapy of Human Filariasis by the Administration of Neostibosan, *Am. J. Trop. Med.* 25: 403-406, 1945.
5. Culbertson, J., Rose, H., and Oliver-Gonzalez, J.: Chemotherapy of Filariasis Due to *Wuchereria Bancrofti* With Neostibosan, *Am. J. Hyg.* 43: 145-151, 1946.
6. Johnstone, H.: The Chemotherapy of *Dirofilaria Immitis*, *Am. J. Trop. Med.* 16: 207-224, 1936.
7. Wright, W., and Underwood, P.: Fouadin in the Treatment of Infestations With the Dog Heartworm, *Dirofilaria Immitis*, *Vet. Med.* 29: 234-246, 1934.
8. Wright, W., and Underwood, P.: A Survey of 1000 Dog Heartworm Cases Treated With Fouadin, *N. Am. Vet.* 17: 19-43, 1936.
9. Otto, G., and Maren, T.: Possible Use of an Arsenical Compound in the Treatment of Heartworm in Dogs, *Vet. Med.* 52: 128, 1947.
10. Cranston, E., Cuckler, A., Litchfield, J., Jr., Brey, T., Wright, H., and Bieter, R.: Chemotherapeutic Activity of Cyanines and Related Compounds in the Cotton Rat, *Federation Proc.* 6: 318, 1947.
11. Welch, A., Peters, L., Bueding, E., Valk, A., Jr., and Higashi, A.: Studies on the Relative Antifilarial Activity of a Series of Cyanine Dyes Against *Litomosoides Carinii*, *Federation Proc.* 6: 382-383, 1947.
12. Culbertson, J., and Rose, H.: Chemotherapy of Filariasis in the Cotton Rat by Administration of Neostam and Neostibosan, *J. Pharmacol. & Exper. Therap.* 81: 181-196, 1944.
13. Culbertson, J., and Price, E.: Chemotherapy of Filariasis (*Litomosoides Carinii*) in the Cotton Rat by the Administration of Stibanose (Solustibosan), *J. Pharmacol. & Exper. Therap.* 87: 181-184, 1946.
14. Williams, R., and Brown, H. W.: The Development of *Litomosoides Carinii*, Filarid Parasite of the Cotton Rat, in the Tropical Rat Mite, *Science* 102: 482-483, 1945.
15. Williams, R., and Brown, H. W.: The Transmission of *Litomosoides Carinii*, Filarid Parasite of the Cotton Rat, by the Tropical Rat Mite, *Liponyssus Bacoti*, *Science* 103: 224, 1946.
16. Bertram, D., Unsworth, K., and Gordon, R.: The Biology and Maintenance of *Liponyssus Bacoti* Hirst, 1913, and an Investigation Into Its Role as a Vector of *Litomosoides Carinii* to Cotton Rats and White Rats, Together With Some Observations on the Infection in White Rats, *Ann. Trop. Med.* 40: 228-254, 1946.

17. Brown, H. W., and Williams, R.: A Method for Counting the Microfilariae of *Litomosoides Carinii* of the Cotton Rat, *Am. J. Trop. Med.* 25: 67-69, 1945.
18. Bell, S., and Brown, H. W.: Studies on the Microfilariae Periodicity of *Litomosoides Carinii*, Filariid Parasite of the Cotton Rat, *Am. J. Trop. Med.* 25: 137-140, 1945.
19. Scott, J.: Observations on the Rate of Growth and Maturity of *Litomosoides Carinii*, a Filarial Worm of the Cotton Rat, *J. Parasitol.* 32: 570-573, 1946.
20. Lawton, A., Brady, F., Ness, A., and Haskins, W.: Tests of Mercury and Antimony Compounds in *Dirofilaria immitis* and *Litomosoides Carinii* Infections, *Am. J. Trop. Med.* 25: 263-269, 1945.
21. Hewitt, R., White, E., Wallace, W., Stewart, H., Kushner, S., and SubbaRow, Y.: Experimental Chemotherapy of Filariasis. II. Effect of Piperazine Derivatives Against Naturally Acquired Filarial Infections in Cotton Rats and Dogs, *J. LAB. & CLIN. MED.* 32: 1304, 1947.
22. Hewitt, R., Kushner, S., Stewart, H., White, E., Wallace, W., and SubbaRow, Y.: Experimental Chemotherapy of Filariasis. III. Effect of 1-Diethylcarbamyl-4-methyl-piperazine Hydrochloride Against Naturally Acquired Filarial Infections in Cotton Rats and Dogs, *J. LAB. & CLIN. MED.* 32: 1314, 1947.
23. Welch, A., Peters, L., Bueding, E., Valk, A., Jr., and Higashi, A.: A New Class of Antifilarial Compounds, *Science* 105: 486-488, 1947.

## EXPERIMENTAL CHEMOTHERAPY OF FILARIASIS

### II. EFFECT OF PIPERAZINE DERIVATIVES AGAINST NATURALLY ACQUIRED FILARIAL INFECTIONS IN COTTON RATS AND DOGS

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#### INTRODUCTION

IN THE preceding paper<sup>1</sup> it was mentioned that compound 180-C (1-carbethoxy-4-methylpiperazine hydrochloride) produced a reduction in microfilariae when administered orally or intraperitoneally to filaria-infected cotton rats, and that suggestive action was produced against the adult worms. The effect of this compound, on the microfilariae at least, was so much more rapid than had been reported for any other known compound that an investigation of other derivatives was indicated. The organic chemistry departments of the Lederle Laboratories Division and the Calco Chemical Division of American Cyanamid Company therefore prepared a number of piperazine compounds for testing in the cotton rat. The results of these tests are given in this paper, and the synthetic part of the program is reported elsewhere.<sup>2, 3</sup>

#### EXPERIMENTAL METHODS

In addition to the general procedures reported in a previous paper<sup>1</sup> for evaluating the effects of new compounds against filariasis in the cotton rat, supplementary methods were used in the case of the piperazines. Several members of this group of compounds were found to produce precipitous reductions in the microfilarial count following various oral or intraperitoneal doses. For comparative evaluations it was necessary therefore to select what appeared to be the most active derivative and to use this compound as a standard, particularly since no other known filaricidal compounds produced comparable effects. During the first few months of this study, 1-carbethoxy-4-methylpiperazine hydrochloride (compound 180-C) produced the most consistent effect against microfilariae and was used as a standard. Data from eighty-one cotton rats treated with various doses of 180-C are given in Table IV.

Although compound 180-C was markedly effective against microfilariae in well-tolerated doses in the cotton rat, the results obtained against adult worms (Table V), although suggestive, were not promising. Moreover, doses which reduced the microfilariae of *Dirofilaria immitis* in the dog frequently caused nausea, muscular weakness, profuse salivation, and prostration. Another compound was subsequently chosen as a standard. This was 1-diethylcarbamyl-4-methylpiperazine hydrochloride (84-L). Marked reductions in microfilariae were produced by this compound in doses as low as 3 mg. per kilogram in cotton rats; no serious toxic symptoms occurred in dogs in therapeutic doses; and by frequent administration a large proportion of the adult worms were killed in cotton rats. A detailed report on the effects produced by 84-L in cotton rats and dogs is given in another paper in this series,<sup>4</sup> but for comparative purposes some data regarding its activity are included herein.

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In testing new piperazine derivatives three rats were used for a preliminary assay. If the compound was soluble, intraperitoneal doses of 50, 25, and 12.5 mg. per kilogram were administered twice daily for from two to four weeks. Insoluble derivatives were administered orally in a 2.5 per cent starch suspension at 200, 100, and 50 mg. per kilogram. If preliminary assays proved the compounds to be toxic in the doses used, second or third trials were made, halving the doses each time. Autopsies were performed within one to four weeks after cessation of treatment.

In the case of piperazines which showed activity against microfilariae several dosage regimes were used and an attempt was made to determine an approximate chemotherapeutic index (Table II).

In several cases, due to a difficult synthesis, relatively small amounts of compounds were available. If these showed no activity in doses which were two or three times greater than the minimum effective dose of 84-L no further tests were performed. It is quite possible, therefore, that some of the compounds listed as inactive in Table I might have shown activity at higher doses.

*Types of Piperazines Tested.*—In Tables I and II are listed most of the piperazines and related compounds which have been tested thus far in cotton rats. Several other piperazines, some of which were active, will be reported in a later publication. The piperazine nucleus itself showed no activity nor did any of its components. The addition of the carbethoxy radical in position 1, with various substitutions in position 4, produced a number of compounds with high microfilaricidal activity. Alkyl radicals in position 4 retained high activity as far as the propyl and isopropyl derivatives, but butyl substitutions were much less active. Toxicity was increased as the alkyl chain was lengthened.

Retaining the carbethoxy group in position 1, two other substitutions in position 4 retained activity, namely guanyl and carbethoxy.

The only compounds lacking the carbethoxy group which showed marked activity against microfilariae were 1-ethylcarbamy-4-methylpiperazine hydrochloride (182-L); 1-diisopropylcarbamy-4-methylpiperazine hydrochloride (177-L); 1-dimethylcarbamy-4-methylpiperazine hydrochloride (152-L); and 1-Diethylcarbamy-4-methylpiperazine hydrochloride (84-L). The latter two compounds are considered as the most active of the piperazines yet tested. †

*Effect of Piperazines on Microfilariae in the Cotton Rat.*—The piperazines listed in Tables II and III produced rapid reductions in microfilariae in the peripheral blood of cotton rats. In some cases the amount of drug necessary to produce this effect was very close to the lethal dose, whereas in others it was far beneath the lethal dose. In general active piperazines in proper doses produced a measurable reduction in microfilariae within twenty-four hours (Table III) after the first dose was administered, and this reduction was sustained for as long as treatment was continued. Recurrences of microfilariae frequently occurred after cessation of treatment.

An exhaustive study of all active piperazines has not been made thus far, since the number of animals and amount of drug necessary to carry out such a study would have been prohibitive, from the standpoint of both time and economy. The comparison given in Tables II and III, however, illustrate the effects of eighteen active piperazines, and Table IV illustrates the effects of different dosage upon microfilariae, using 1-carbethoxy-4-methylpiperazine hydrochloride.

TABLE I. PIPERAZINE AND RELATED COMPOUNDS SHOWING NO FILARICIDAL ACTIVITY IN COTTON RATS IN THE DOSES USED

COMPOUND	NAME	HIGHEST* DOSAGE USED (MG./KG.)
148-L	Ethyl carbamate	100 I.P.
150-L	Ethyl N-methylcarbamate	100 I.P.
149-L	Ethyl N-ethylcarbamate	100 I.P.
146-L	Ethyl N,N-diethylcarbamate	100 I.P.
144-L	N-carbethoxyethylenediamine	100 I.P.
220-C	Ethyl N-ethyl-N-(2-hydroxyethyl)-carbamate	100 I.P.
U-930	Piperazine hexahydrate	800 I.P.
63-L	1-Phenylpiperazine hydrochloride	100 I.P.
56-L	1-Ethylpiperazine hydrochloride	100 I.P.
U-910	1,4-Diaminopiperazine hydrochloride	100 I.P.
222-C	1,4-Dimethylpiperazine dihydrochloride	100 I.P.
225-C	1-Carbamyl-4-methylpiperazine	100 I.P.
228-C	1-Methyl-4-thiocarbamylpiperazine hydrochloride	100 I.P.
309-C	Omega-[1-(4-methylpiperazyl)]-propiophenone dihydrochloride	12.5 I.P.
240-C	2,3-Diphenylpiperazine dihydrochloride	100 I.P.
77-L	1,4-Diguanylpiperazine sulfate	100 I.P.
214-C	4-Carbethoxymorpholine	200 Orally
184-L	1-Carbomethoxy-4-methylpiperazine hydrochloride	6.25 I.P.
U-994	1-Carbethoxy-4-nitrosopiperazine hydrochloride	50 Orally
145-L	1-Carbethoxy-4-isobutylpiperazine	100 Orally
224-C	1-Carbethoxy-4-(n-heptyl)-piperazine	200 Orally
193-C	1-Carbethoxy-4-phenylpiperazine	25 I.P.
209-C	1-Carbethoxy-4-benzylpiperazine hydrochloride	200 Orally
60-L	1-Carbethoxy-4-aminopiperazine hydrochloride	100 I.P.
219-C	1-Carbamyl-4-carbethoxypiperazine	100 I.P.
211-C	1-Carbethoxy-4-allylpiperazine	100 I.P.
192-L	4-( $\beta$ -sulfanylethyl)-1-carbethoxypiperazine	200 Orally
195-L	1-Diethylcarbamyl-4-carbethoxypiperazine	12.5 I.P.
217-C	1,2-Bis-(1-carbethoxy-4'-piperazine) ethane	200 Orally
196-C	1-Ethyl 4[2-(...)-piperazine	100 I.P.
153-L	1,1'-Carbonyl-1... dihydrochloride	100 I.P.
168-C	1,4-Bis-( $\alpha$ -phenyl- $\beta$ -benzoylethyl)-piperazine	100 Orally
U-683	Ethyl (1-carbethoxy-4-piperazine)-acetate	100 I.P.
U-682	1-Carbethoxy-4-(2-hydroxyethyl)-piperazine	100 I.P.
162-L	1-Carbomethoxypiperazine	6.25 I.P.
164-L	1-Carbobutoxypiperazine	6.25 I.P.
165-L	1-Carboisobutoxypiperazine	6.25 I.P.
161-L	1,4-Dicarbomethoxypiperazine	6.25 I.P.
163-L	1,4-Dicarbobutoxypiperazine	25 Orally
166-L	1,4-Dicarboisobutoxypiperazine	6.25 I.P.
159-L	1-Diethylcarbamylpiperazine	6.25 I.P.
197-L	1-Diisopropylcarbamylpiperazine	25 I.P.
198-L	1-Di-n-butylcarbamylpiperazine	25 I.P.
199-L	1-Diisoamylcarbamylpiperazine	25 I.P.
176-L	1-Dibutylcarbamyl-4-methylpiperazine hydrochloride	6.25 I.P.
203-L	1-Phenylcarbamyl-4-methylpiperazine	25 I.P.
293-L	1-Benzylcarbamyl-4-methylpiperazine	25 I.P.
158-L	1-Diethylcarbamyl-4-isopropylpiperazine hydrochloride	6.25 I.P.
160-L	1,4-Bis-diethylcarbamylpiperazine	6.25 I.P.

\*Treatment administered twice daily for from two to four weeks.

More extensive data concerning the effects of 1-diethylcarbamyl-4-methylpiperazine hydrochloride (84-L) upon microfilariae in vivo and in vitro are presented elsewhere.<sup>4</sup>

*Effect of Piperazines on Adult Filariae in Cotton Rats.*—In cotton rats treated with several of the piperazines listed in Tables II and III, autopsies performed at various intervals after cessation of treatment revealed no dead

TABLE II. COMPARATIVE MICROFILARICIDAL ACTIVITY AND TOXICITY OF EIGHTEEN PIPERAZINE DERIVATIVES IN COTTON RATS, USING 1-DIETHYLCARBAMYL-4-METHYLPYPERAZINE HYDROCHLORIDE (S1-L) AS A STANDARD

COM- POUND	NAME	MINIMUM EFFECTIVE DOSE AGAINST MICRO- FILARIAE (MG./KG.)	LD <sub>50</sub> MICE I.P. (MG./KG.)	APPROXI- MATE 84-L EQUIVA- LENT	APPROXI- MATE CHEMO- THERA- PEUTIC INDEX
163-C	1-Phenylpiperazine hydrochloride	50 I.P.	140	0.06	2.8
162-C	1-Carbethoxypiperazine hydrochloride	25 I.P.	275	0.125	11.0
217-L	1-Methyl-4-(4'-morpholine carbamyl)-piperazine hydrochloride	12.5 I.P.	?	0.25	?
180-C	1-Carbethoxy-4-methylpiperazine hydrochloride	6.25 I.P.	550	0.5	88.0
59-L	1-Carbethoxy-4-ethylpiperazine	50 I.P.	175	0.06	3.5
82-L	1-Carbethoxy-methylpiperazine	100 I.P.	?	0.03	?
U-653	1-Carbethoxy-4-propylpiperazine hydrochloride	25 I.P.	87.5	0.125	3.5
147-L	1-Carbethoxy-4-isopropylpiperazine hydrochloride	25 I.P.	100	0.125	4.0
U-655	1-Carbethoxy-4-butylpiperazine	50 I.P.	125	0.06	2.5
U-801	1-Carbethoxy-4-s. butylpiperazine	100 Orally	175	0.05	1.75*
61-L	1-Guanyl-4-carbethoxy-piperazine sulfate	50 I.P.	285	0.06	5.6
218-C	Bis-(4-carbethoxy-1-piperazine)-methane	25 I.P.	175	0.125	7.0
169-C	1,4-Dicarbethoxypiperazine	100 I.P.	500	0.03	5.0
76-L	1,4-Dicarbethoxy-2-methylpiperazine	200 Orally	375	0.025	1.9*
182-L	1-Ethylcarbamyl-4-methylpiperazine hydrochloride	6.25 I.P.	2,250	0.5	360.0
152-L	1-Dimethylcarbamyl-4-methylpiperazine hydrochloride	6.25 I.P.	310	0.5 to 1.0	48.0
177-L	1-Diisopropylcarbamyl-4-methylpiperazine hydrochloride	6.25 I.P.	160	0.5	25.6
84-L	1-Diethylcarbamyl-4-methylpiperazine hydrochloride	3.13 I.P.	285	1.0	91.0

\*Probably not a true value, since the LD<sub>50</sub> was determined by intraperitoneal injections and the minimum effective dose by oral administration.

adult worms present in the pleural cavity, and there was no macroscopic or microscopic evidence that any damage whatever to the macrofilariae had been effected. In view of the dramatic effects produced against the microfilariae these results were at first disappointing.

One of the first compounds found to produce suggestive activity against the adult worms was 1-carbethoxy-4-isopropylpiperazine hydrochloride (147-L). Dead worms were found in one of the three rats first used to test this compound, and no adult worms were found in another. The third rat revealed all living worms. Attempts to repeat these results in a larger series of rats failed, because this compound was very toxic to the host and prolonged dosage could not be administered.

Thirty rats treated with various doses of 1-carbethoxy-4-methylpiperazine hydrochloride were then brought to autopsy, and, as illustrated in Table V,

TABLE III. EFFECT OF VARIOUS PIPERAZINE DERIVATIVES AGAINST MICROFILARIAE IN COTTON RATS DURING FIRST WEEK OF TREATMENT

COM- POUND	NAME	DOSE (MG./ KG.) R.I.D.	MICROFILARIAE PER 100 FIELDS						
			DAY						
			1	2	3	4	5	6	7
163-C	1-Phenylpiperazine hydrochloride	25 I.P. 310 50 I.P. 92	310 8	284 8	310 0	110 60	111 18	250 24	108 0
162-C	1-Carbethoxypiperazine hydrochloride	25 I.P. 180 50 I.P. 652 100 I.P. 1,360	180 252 81	6 252 8	4 96 8	6 18 10	6 14 4	8 14 6	6 12 6
217-L	1-Methyl-4-(4'-morpho- line carbamyl-pipera- zine hydrochloride	25 I.P. 268 50 I.P. 11 100 I.P. 92	268 11 92			10 6			2 1 0
180-C	1-Carbethoxy-4-methyl- piperazine hydrochlo- ride	6.25 I.P. 430 12.5 I.P. 128 25 I.P. 160 50 I.P. 384 100 I.P. 264 200 I.P. 168	430 128 160 384 264 168	190 72 112 40 26 12	56 60 28 22 8 2	250 48 38 12 2 1	52 22 10 4 6 1	8 12 6 0 8 4	22 56 14 4 0 0
59-L	1-Carbethoxy-4-ethyl- piperazine	25 I.P. 1,580 50 I.P. 1,910 100 I.P. 136	1,580 1,910 136	800 600 6			430 188 0		360 100 1
82-L	1-Carbethoxy-methyl- piperazine	25 I.P. 110 50 I.P. 212 100 I.P. 76	110 212 76	144 320 14	132 124 18	116 124 14	248 156 12	308 136 10	140 84 6
U-653	1-Carbethoxy-4-propyl- piperazine hydrochlo- ride	25 I.P. 64 50 I.P. 248	64 248	28 160	22 36		32 18		14 14
147-L	1-Carbethoxy-4-isopro- pylpiperazine hydro- chloride	25 I.P. 44 50 I.P. 26 100 I.P. 8	44 26 8	3 6 0	10 1 0		4 4 0		1 8 0
U-655	1-Carbethoxy-4-butyl- piperazine	25 I.P. 284 50 I.P. 160 100 I.P. 116	284 160 116	656 18 10	696 10 9		208 6 10		336 12 4
U-801	1-Carbethoxy-4-s. butyl- piperazine	50 Orally 388 100 Orally 140 200 Orally 410	388 140 410	168 80 44	148 26 8		368 28 24		340 14 0
61-L	1-Guanyl-4-carbethoxy- piperazine sulfate	25 I.P. 380 50 I.P. 388 100 I.P. 104	380 388 104	212 68 12		136 18 6	272 4 8	412 24 6	304 6 8
218-C	Bis-(4-carbethoxy-1- piperazine)-methane	25 I.P. 272 50 I.P. 76	272 76	68 32	60 6				22 3
169-C	1,4-Dicarbethoxypipera- zine	12.5 I.P. 132 25 I.P. 188 50 I.P. 440 100 I.P. 344 200 I.P. 740	132 188 440 344 740	196 140 552 88 132	116 140 176 38 30	172 60 676 22 6	40 23 318 56 18	120 48 118 36 6	88 128 188 26 34
76-L	1,4-Dicarbethoxy-2- methylpiperazine	50 Orally 568 100 Orally 292 200 Orally 52	568 292 52	400 292 61	248 412 38	92 76 10	320 72 6	400 268 2	160 116 8
182-L	1-Ethylcarbamyl-4- methylpiperazine hydrochloride	1.5 I.P. 188 3.13 I.P. 432 6.25 I.P. 36	188 432 36	332 1,060 72	188 324 48	232 172 28	176 712 4	76 490 10	244 612 6
152-L	1-Dimethylcarbamyl-4- methylpiperazine hydrochloride	25 I.P. 80 50 I.P. 1,190 100 I.P. 172	80 1,190 172	20 2,180 60	2 740 8	3 870 12	6 960 6	4 560 4	4 310* 1
177-L	1-Diisopropylcarbamyl- 4-methylpiperazine hydrochloride	1.5 I.P. 152 3.13 I.P. 432 6.25 I.P. 60	152 432 60	28 104 1	380 136 20	10 336 10	104 244 6	26 180 2	

\*No adult worms were found in the pleural cavity at autopsy. Four worms were found in the abdominal cavity.

TABLE III—CONT'D

COM- POUND	NAME	DOSE (MG./ KG.) B.I.D.	MICROFILARIAE PER 100 FIELDS						
			DAY						
			1	2	3	4	5	6	7
84-L	1 Diethylcarbamyl 4-methylpiperazine hydrochloride	1.5 I.P.	180	100	310	270	290	210	181
		3.13 I.P.	1,220	108	10	56	4	8	2
		6.25 I.P.	600	116	11	22	72	10	22
		12.5 I.P.	180	61	8	8	8	1	8
		25 I.P.	396	100	20	8	10	6	1
		50 I.P.	176	76	22	13	12	2	3
		100 I.P.	192	12	6	5	1	10	1
		5 Orally	228	11	0	10	3	2	1
		10 Orally	108	10	6	10	3	2	1
		25 Orally	168	8	0	0	0	0	0
	Nontreated controls	—	1,630	252	590	1560	224	1020	1180
			16	20	28	18	44	40	72
			36	26	56	41	52	58	36
			10	60	88	22	40	21	56
			560	212	208	181	280	244	410
			120	248	318	100	404	628	780
			10	24	24	10	12	42	56

the effects upon the adult worms were extremely variable. Most of the adult worms were alive, however. Similar results were obtained on a smaller series of animals treated with other piperazines effective against microfilariae.

Nine of sixty-five untreated rats which were autopsied showed one or two dead adult worms at autopsy, but in no case were the majority or all of the worms dead. The effects produced by several of the piperazines upon the adult worms in a few rats in each series, therefore, suggested that a wide variety of dosage schedules, with autopsies at different periods after cessation of treatment, might throw some light on the amount of drug and the length of time necessary to kill a larger proportion of the adult worms than had been accomplished in earlier trials. The compound selected for these extensive tests was 1-diethylcarbamyl-4-methylpiperazine hydrochloride, since it produced predictable and rapid reductions in microfilariae in oral or intraperitoneal doses as low as 3 mg. per kilogram, was relatively nontoxic to cotton rats, and was made available in large quantities.

Subsequent data obtained with this compound and reported in a following paper showed that frequent oral dosage for several weeks killed a large proportion of the adult worms. None of the other compounds listed in Tables II and III have been studied so extensively, but it is believed that the best balance between activity and toxicity has been achieved with 1-diethylcarbamyl-4-methylpiperazine hydrochloride. For the reasons described, the activity of the piperazines listed in Tables II and III is based entirely upon their effectiveness against microfilariae. In most cases relatively high toxicity to the host rules out further studies upon their effectiveness against adult worms, and in the few instances where good effects against microfilariae were obtained with relatively nontoxic doses (182-L, 152-L, 180-C) it is felt that further studies would produce little more pertinent information than has been obtained with 84-L.

*Effect of Piperazines Upon Dirofilaria Immitis in the Dog.*—Several piperazines showing high activity in the cotton rat were tested for effectiveness against

TABLE IV. EFFECT OF 1-CARBETHOXY-4-METHYLPYPERAZINE HYDROCHLORIDE (180-C) AGAINST MICROFILARIAE IN COTTON RATS

RAT	SERIES	DOSE (MG./KG.)	TREATMENT	PER CENT REDUCTION OF MICROFILARIAE	
				7 DAYS	LAST DAY OF TREATMENT
370	RF-32	12½	I.P., b.i.d., 14 days	56.3	59.4
44	RF-9	25	I.P., b.i.d., 14 days	97.5	100.0
38	RF-9	25	I.P., b.i.d., 14 days	70.0	90.0
75	RF-9	25	I.P., b.i.d., 14 days	100.0	100.0
109	RF-9	25	I.P., b.i.d., 14 days	92.3	94.3
70	RF-9	25	I.P., b.i.d., 14 days	25.0	75.0
76	RF-9	25	I.P., b.i.d., 14 days	95.6	94.0
82	RF-9	25	I.P., b.i.d., 14 days	97.8	77.5
133	RF-10	25	I.P., b.i.d., 27 days	100.0	91.7
134	RF-10	25	I.P., b.i.d., 27 days	94.5	77.8
135	RF-10	25	I.P., b.i.d., 27 days	100.0	D.
137	RF-10	25	I.P., b.i.d., 27 days	100.0	14.3
138	RF-10	25	I.P., b.i.d., 27 days	50.0	62.5
372	RF-32	25	I.P., b.i.d., 14 days	91.3	93.8
373	RF-32	25	I.P., b.i.d., 14 days	74.0	88.1
205	RF-16	25	Orally, b.i.d., 28 days	99.6	99.1
210	RF-16	25	Orally, b.i.d., 28 days	99.4	D.
215	RF-16	25	Orally, b.i.d., 28 days	99.4	D.
218	RF-16	25	Orally, b.i.d., 28 days	98.5	99.4
222	RF-16	25	Orally, b.i.d., 28 days	98.2	D.
139	RF-10	50	I.P., b.i.d., 27 days	100.0	100.0
140	RF-10	50	I.P., b.i.d., 27 days	50.0	87.5
141	RF-10	50	I.P., b.i.d., 27 days	83.4	100.0
142	RF-10	50	I.P., b.i.d., 27 days	87.5	100.0
143	RF-10	50	I.P., b.i.d., 27 days	100.0	D.
159	RF-10	50	I.P., b.i.d., 27 days	100.0	100.0
160	RF-10	50	I.P., b.i.d., 27 days	99.4	100.0
374	RF-32	50	I.P., b.i.d., 14 days	100.0	D.
375	RF-32	50	I.P., b.i.d., 14 days	99.0	99.0
207	RF-16	50	Orally, b.i.d., 28 days	100.0	99.3
209	RF-16	50	Orally, b.i.d., 28 days	99.4	D.
216	RF-16	50	Orally, b.i.d., 28 days	95.0	98.8
219	RF-16	50	Orally, b.i.d., 28 days	97.7	98.5
183	RF-15	50	I.P., O.D., 28 days	96.3	100.0
189	RF-15	50	I.P., O.D., 28 days	92.9	100.0
190	RF-15	50	I.P., O.D., 28 days	100.0	96.6
184	RF-15	50	I.P., A.D., 28 days	94.7	D.
187	RF-15	50	I.P., A.D., 28 days	93.4	96.7
197	RF-15	50	I.P., A.D., 28 days	20.0	0
144	RF-10	100	I.P., b.i.d., 14 days	95.0	80.0
145	RF-10	100	I.P., b.i.d., 14 days	99.0	99.0
146	RF-10	100	I.P., b.i.d., 14 days	100.0	D.
148	RF-10	100	I.P., b.i.d., 14 days	100.0	D.
149	RF-10	100	I.P., b.i.d., 14 days	100.0	100.0
376	RF-32	100	I.P., b.i.d., 14 days	97.6	100.0
385	RF-32	100	I.P., b.i.d., 14 days	98.4	100.0
56	RF-11	100	I.P., b.i.d., 29 days	100.0	98.8
131	RF-11	100	I.P., b.i.d., 29 days	100.0	99.6
90	RF-11	100	I.P., b.i.d., 29 days	100.0	100.0
206	RF-16	100	Orally, b.i.d., 28 days	97.3	100.0
208	RF-16	100	Orally, b.i.d., 28 days	98.9	D.
213	RF-16	100	Orally, b.i.d., 28 days	98.8	98.8
221	RF-16	100	Orally, b.i.d., 28 days	98.4	D.
186	RF-15	100	I.P., O.D., 28 days	87.0	78.3
196	RF-15	100	I.P., O.D., 28 days	98.1	94.3
212	RF-15	100	I.P., O.D., 28 days	99.0	100.0
194	RF-15	100	I.P., A.D., 28 days	92.3	69.3
203	RF-15	100	I.P., A.D., 28 days	90.5	54.1
204	RF-15	100	I.P., A.D., 28 days	87.0	98.2
155	RF-19	100	*	D.	D.

TABLE IV—CONT'D

RAT	SERIES	DOSE (MG./KG.)	TREATMENT	PER CENT REDUCTION OF MICROFILARIAE	
				7 DAYS	LAST DAY OF TREATMENT
153	RF-19	100	*	98.3	98.3
157	RF-19	100	*	100.0	100.0
158	RF-19	100	*	D.	D.
98	RF-11	200	I.P., b.i.d., 29 days	D.	D.
124	RF-11	200	I.P., b.i.d., 29 days	100.0	100.0
102	RF-11	200	I.P., b.i.d., 29 days	100.0	100.0
386	RF-32	200	I.P., b.i.d., 14 days	98.0	100.0
387	RF-32	200	I.P., b.i.d., 14 days	100.0	99.3
18	RF-11	200	Orally, b.i.d., 29 days	99.3	100.0
33	RF-11	200	Orally, b.i.d., 29 days	99.2	D.
40	RF-11	200	Orally, b.i.d., 29 days	100.0	D.
248	RF-19	200	*	97.8	97.8
250	RF-19	200	*	100.0	100.0
251	RF-19	200	*	100.0	100.0
252	RF-19	200	*	100.0	100.0
50	RF-11	400	Orally, b.i.d., 29 days	100.0	D.
55	RF-11	400	Orally, b.i.d., 29 days	100.0	100.0
127	RF-11	400	Orally, b.i.d., 29 days	100.0	D.
88	RF-12	Diet 0.5%	15 days	97.5	98.4
92	RF-12	Diet 0.5%	15 days	100.0	98.9
96	RF-12	Diet 0.5%	15 days	91.6	D.

I.P., Intraperitoneal b.i.d., twice daily; O.D., once daily; A.D., once on alternate days; D., death.

\*Series RF-19 treated every two hours for forty-eight hours, then twice daily for six more days.

*Dirofilaria immitis* in the dog. The only two compounds which produced measurable reductions in microfilariae in tolerated doses were 1-carbethoxy-4-methylpiperazine hydrochloride (180-C) and 1-diethylcarbamy-4-methylpiperazine hydrochloride (84-L). The administration of oral or intraperitoneal doses of compound 180-C which produced reductions of microfilariae (25 to 100 mg. per kilogram twice daily) invariably produced nausea, profuse salivation, and muscular weakness. Compound 84-L was much better tolerated, and subsequent experiments in dogs were confined to this derivative. The results obtained with 84-L in dogs, against both micro- and macrofilariae, are presented in another paper in this series.

#### DISCUSSION

The effectiveness of several piperazine derivatives (administered orally or intraperitoneally) in rapidly reducing the microfilarial count in filaria-infected cotton rats is unique in the experimental chemotherapy of this infection. If the effectiveness of the piperazines was confined to the microfilariae, it would probably indicate that their potential usefulness for treating filarial diseases in other animals and man would be limited. The fact that several of the piperazines tested showed suggestive action against the adult worms, and that one in particular killed a large proportion of the adult worms when administered frequently in tolerated doses for several weeks, certainly removes this group of compounds from the class of experimental curiosities.

The value of the cotton rat bio-assay method for predicting potential filaricidal activity against related diseases in human beings has not been fully tested.

TABLE V. EFFECT OF 1-CARBETHOXY-4-METHYLPIPERAZINE HYDROCHLORIDE AGAINST ADULT WORMS IN COTTON RATS

RAT	DOSE (MG./KG.)	TREATMENT	MICROFILARIAE PER 100 FIELDS			AUTOPSY (DAYS AFTER LAST TREAT- MENT)	AUTOPSY RECORDED
			DAY 1	DAY 7	DAY OF AUTOPSY		
117	25 I.P.	b.i.d., 7 days	24	4	84	15	Mass of live worms
119	25 I.P.	b.i.d., 7 days	31	0	104	15	Mass of live worms
38	25 I.P.	b.i.d., 14 days	40	6	4	1	Several dead worms; none alive
75	25 I.P.	b.i.d., 14 days	30	0	0	1	Several live and sev- eral dead worms
109	25 I.P.	b.i.d., 14 days	52	4	2	1	Several live and sev- eral dead worms
70	25 I.P.	b.i.d., 14 days	56	42	8	1	Several live and sev- eral dead worms
76	25 I.P.	b.i.d., 14 days	68	3	4	1	Several live and sev- eral dead worms
82	25 I.P.	b.i.d., 14 days	132	3	4	1	Mass of live worms
135	25 I.P.	b.i.d., 10 days	18	0	0	1	Several live and sev- eral dead worms
143	50 I.P.	b.i.d., 15 days	4	0	0	1	Mass of live worms
146	100 I.P.	b.i.d., 19 days	8	0	0	1	Mass of live worms
141	50 I.P.	b.i.d., 26 days	6	1	0	13	Several live worms
40	200 Orally	b.i.d., 11 days	200	0	4	3	Mass of live worms
90	100 I.P.	b.i.d., 28 days	64	0	424	55	Mass of dead worms; none alive
18	200 Orally	b.i.d., 28 days	132	1	0	8	Several dead worms; none alive
55	400 Orally	b.i.d., 28 days	356	0	40	8	Mass of live worms
215	25 I.P.	b.i.d., 12 days	146	3	2	1	Mass of live worms
209	50 Orally	b.i.d., 13 days	160	1	0	1	Mass of live worms
208	100 Orally	b.i.d., 11 days	348	4	0	1	Mass of live worms
195	1.5 I.P.	b.i.d., 14 days	740	76	428	56	Mass of live worms
198	3.13 I.P.	b.i.d., 14 days	1,840	248	192	56	Mass of live worms
41	6.25 I.P.	b.i.d., 14 days	430	22	52	56	Mass of live worms
104	12.5 I.P.	b.i.d., 14 days	104	16	184	56	Mass of live worms
539	5 Orally	b.i.d., 10 days	1,700	180	528	1	Mass of live worms
527	5 I.P.	b.i.d., 15 days	72	172	2	112	Mass of live worms
522	5 I.P.	b.i.d., 15 days	252	80	380	112	Mass of live worms
532	5 I.P.	b.i.d., 15 days	26	6	84	112	Mass of live worms
530	5 Orally	b.i.d., 15 days	1,080	370	630	112	Mass of live worms
529	5 Orally	b.i.d., 15 days	204	40	24	112	Mass of live worms
540	5 Orally	b.i.d., 15 days	600	112	212	112	Mass of live worms

Culbertson, Rose, and Olive-Gonzalez,<sup>5-7</sup> however, have demonstrated that a correlation exists between the effects produced by Neostibosan against *Litomosoides carinii* in cotton rats and *Wuchereria bancrofti* infections in man.

We believe it to be important, also, that several of the most active piperazines are effective when administered orally. With the exception of malaria and the intestinal helminth diseases, the chemotherapy of parasitic diseases in man is largely confined to the parenteral administration of various drugs, usually compounds containing antimony or arsenic. The value of some of the heavy metals in the treatment of several of the most important parasitic diseases is not disputed, but the advantages of an effective oral treatment for any disease in human beings or domestic animals are obvious, particularly if no toxic effects are produced.



There is a measurable correlation between structure and microfilaricidal activity in the piperazines, as demonstrated in Table II. We cannot be sure that the most active member of the group has been found, and there is room for further investigation in this respect.

In comparison with several antimony derivatives, the piperazines produce a much more rapid effect upon the microfilariae of cotton rats in the peripheral blood.<sup>8,9</sup> The effect produced against adult worms by piperazines is very slow in comparison with that of antimony salts. An entirely different mode of action must therefore be involved. No studies have been made thus far in attempts to determine how the piperazines produce their effects upon either microfilariae or macrofilariae.

#### SUMMARY

The effects of various piperazine derivatives against naturally acquired filaria infections in cotton rats and dogs are discussed. Several compounds in this group produce rapid reductions in microfilariae following oral or intra-peritoneal dosage, and one in particular produces a lethal effect against adult filariae when administered frequently for a period of several weeks.

#### REFERENCES

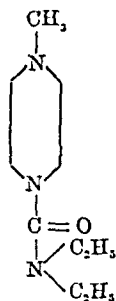
1. Hewitt, R., Wallace, W., White, L., and SubbaRow, Y. Experimental Chemotherapy of Filariasis. I. Experimental Methods for Testing Drugs Against Naturally Acquired Filarial Infections in Cotton Rats and Dogs, *J. LAB & CLIN MED.* 32: 1293, 1947.
2. Stewart, H., Turner, R., Denton, J., Kushner, S., Brancone, L., McEwen, W., Hewitt, R., and SubbaRow, Y. Experimental Chemotherapy of Filariasis. IV The Preparation of Derivatives of Piperazine, *J. Org. Chem.* In press.
3. Kushner, S., Brancone, L., Hewitt, R., McEwen, W., SubbaRow, Y., Stewart, H., Turner, R., and Denton, J. Experimental Chemotherapy of Filariasis. V The Preparation of Derivatives of Piperazine, *J. Org. Chem.* In press.
4. Hewitt, R., Kushner, S., Stewart, H., White, E., Wallace, W., and SubbaRow, Y.: Experimental Chemotherapy of Filariasis. III. Effect of 1 Diethylcarbamyl 4 Methylpiperazine Hydrochloride Against Naturally Acquired Filarial Infections in Cotton Rats and Dogs, *J. LAB & CLIN MED.* 32: 1314, 1947.
5. Culbertson, J., Rose, H., and Oliver Gonzalez, J.: Chemotherapy of Human Filariasis by the Administration of Neostibosan, *Am. J. Trop. Med.* 25: 271-274, 1945.
6. Culbertson, J., Rose, H., and Oliver Gonzalez, J.: The Chemotherapy of Human Filariasis by the Administration of Neostibosan, *Am. J. Trop. Med.* 25: 403-406, 1945.
7. Culbertson, J., Rose, H., and Oliver Gonzalez, J.: Chemotherapy of Filariasis Due to *Wuchereria Bancrofti* With Neostibosan, *Am. J. Hyg.* 43: 145-151, 1946.
8. Culbertson, J., and Rose, H. Chemotherapy of Filariasis in the Cotton Rat by Administration of Neostam and Neostibosan, *J. Pharmacol. & Exper. Therap.* 81: 181-196, 1944.
9. Culbertson, J., and Price, E. Chemotherapy of Filariasis (*Litomosoides Carini*) in the Cotton Rat by the Administration of Stibanose (Solustibosan), *J. Pharmacol. & Exper. Therap.* 87: 181-184, 1946.

## EXPERIMENTAL CHEMOTHERAPY OF FILARIASIS

### III. EFFECT OF 1-DIETHYLCARBAMYL-4-METHYLPYPERAZINE HYDROCHLORIDE AGAINST NATURALLY ACQUIRED FILARIAL INFECTIONS IN COTTON RATS AND DOGS

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IT HAS been demonstrated in a preceding paper<sup>1</sup> that the intraperitoneal or oral administration of various piperazine derivatives to cotton rats naturally infected with *Litomosoides carinii* causes a rapid and precipitous reduction of microfilariae in the peripheral blood. Of more than one hundred derivatives made available, 1-diethylcarbamyl-4-methylpiperazine hydrochloride (compound 84-L) was selected as the most promising because of its repeated marked effects against microfilariae in both cotton rats and dogs and because of its relatively low toxicity in these animals. Subsequent studies showed that proper dosage regimes killed a large percentage of the adult worms. The structural formula of this compound is given below.



Two hundred twelve cotton rats and twenty-five dogs have been used to test the effectiveness of this compound against both microfilariae and macrofilariae, and the results obtained are reported herein. The methods used for counting microfilariae and for evaluating the effect upon adult worms have been discussed in a preceding paper.<sup>2</sup>

*Effect of 1-Diethylcarbamyl-4-Methylpiperazine Hydrochloride Against Microfilariae.*—After oral or intraperitoneal treatment with this compound in filaria-infected cotton rats, at doses ranging from 3 to 100 mg. per kilogram, the microfilarial count drops precipitously within twenty-four hours (Tables I and II). It then becomes negative or remains at a very low level for as long as treatment is continued.

There may or may not be a recurrence of microfilariae in the peripheral blood upon cessation of treatment (Tables III, V, IX, and X). The extent of

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TABLE I. EFFECT OF 84-L ON THE MICROFILARIAE OF COTTON RATS  
(INTRAPERITONEAL DOSAGE)

RAT	DOSE (MG./KG., B.I.D.)	MICROFILARIAE PER 100 LOW-POWER FIELDS								
		DAYS DURING TREATMENT								
		1	2	3	4	5	6	7	14	
566	5	92	10	2	2	4	4	6	4	
579	5	36	14	1	1	1	0	0	4	
571	5	76	8	14	24	22	8	1	1	
584	5	48	12	0	0	0	1	8	0	
565	10	112	10	6	2	0	0	0	0	
583	10	100	52	8	5	22	10	8	6	
588	10	232	56	2	6	1	0	0	0	
562	10	548	28	8	4	4	4	1	0	
567	25	364	30	1	3	1	2	2	1	
572	25	692	20	0	0	4	4	0	1	
587	25	452	8	1	2	2	0	1	1	
548	25	184	36	6	1	1	2	2	0	
568	50	22	14	0	0	4	1	0	0	
574	50	408	22	2	0	4	0	0	0	
542	50	44	26	8	3	6	4	6	1	
586	50	52	2	6	0	1	1	0	0	
552	100	36	22	8	4	4	4	6	0	
576	100	48	6	0	1	2	3	1	0	
580	100	52	30	8	12	44	26	6	0	
582	100	284	24	4	1	0	1	1	4	

TABLE II. EFFECT OF 84-L ON THE MICROFILARIAE OF COTTON RATS (ORAL DOSAGE)

RAT	DOSE (MG./KG., B.I.D.)	MICROFILARIAE PER 100 LOW-POWER FIELDS								
		DAYS DURING TREATMENT								
		1	2	3	4	5	6	7	14	
900	5	48	12	4	5	1	0	1	0	
934	5	228	14	0	10	3	2	4	2	
897	5	248	26	22	4	8	3	8	8	
912	5	152	36	12	20	8	4	2	6	
724	5	68	24	6	6	4	3	0	8	
926	5	140	10	6	10	0	3	22	4	
898	5	56	16	10	4	1	4	0	0	
938	5	92	10	4	4	2	0	2	1	
791	5	570	12	8	2	2	2	1	3	
927	10	44	20	14	6	6	0	2	2	
908	10	408	10	6	10	3	2	1	0	
909	10	56	14	8	6	1	0	1	1	
911	10	400	2	6	6	14	6	4	1	
915	10	12	1	4	6	3	0	0	0	
709	10	10	10	3	4	6	2	2	1	
917	10	1,260	24	4	6	4	4	0	1	
919	10	12	2	0	0	1	1	0	0	
920	10	156	6	6	0	4	1	0	1	
933	10	68	18	3	2	4	2	2	6	
895	25	72	3	0	0	0	0	0	0	
940	25	168	8	0	0	0	0	0	0	
913	25	40	1	2	0	0	1	0	0	
925	25	36	1	0	0	0	0	0	0	
931	25	160	3	4	1	2	1	0	0	
637	25	156	2	1	0	1	0	0	0	
924	25	292	12	1	0	0	0	0	0	
930	25	72	6	0	0	0	1	0	0	
936	25	288	3	0	0	0	2	6	1	
921	25	96	3	0	1	0	0	1	0	
775	25	224	6	10	6	0	0	1	0	
904	25	18	10	0	0	0	0	0	0	
905	25	116	1	0	0	0	1	0	1	

TABLE III. EFFECT OF S4-L AGAINST FILARIASIS IN COTTON RATS (ORAL TREATMENT EVERY TWO HOURS FOR FORTY-EIGHT HOURS)

RAT	DOSE (MG./ KG.)	MICROFILARIAE PER 100 FIELDS						AUTOPSY RECORD
		BEFORE TREAT- MENT	HOURS AFTER TREATMENT			DAYS AFTER TREATMENT		
			2	12	24	4	11	
1284	25	148	3	0	0	-	-	Killed at 48 hr.; large mass of live worms
1286	25	340	4	0	0	-	-	Killed at 48 hr.; large mass of live worms
1293	25	48	4	1	0	-	-	Killed at 48 hr.; large mass of live worms
1268	25	220	34	1	0	-	-	Killed at 48 hr.; large mass of live worms
1278	25	156	3	0	0	1	16	Killed on 12th day; mass of live worms; several dead worms; small clump of caseous material
1292	25	248	22	2	0	2	6	Killed on 12th day; one large clump of worms on ventral side of heart, most of these dead; small mass of worms under lungs, some of these living, others dead
1253	25	320	22	0	0	0	16	Killed on 12th day; small clump of live worms, some encased in caseous material
1297	25	964	24	0	0	0	18	Killed on 12th day; many dead and degenerating worms; some living worms
1259	25	664	10	3	1	6	30	Killed on 12th day; mass of live worms; several dead worms
1256	10	148	10	0	0	Dead	-	Dead at 36 hr.; no autopsy
1266	10	676	8	8	2	4	12	Killed on 12th day; small mass of worms, some dead
1226	10	16	0	0	0	0	-	Killed on 8th day; small mass of live worms; 3 dead worms; some caseous material present
1246	10	400	30	8	22	-	-	Killed at 32 hr.; mass of live worms; two clumps of dead and disintegrating worms
1255	10	532	8	0	0	0	-	Killed on 8th day; masses of live worms; 1 dead worm
1264	10	248	8	0	0	0	16	Killed on 12th day; large mass of worms; many dead
1265	10	604	22	16	4	9	92	Killed on 12th day; large mass of worms; many living and some dead
1274	10	592	104	44	9	-	-	Dead at 42 hr.; large mass of live worms; several small clumps of dead worms
1267	10	748	32	14	1	2	48	Killed on 12th day; small mass of live worms; large clump of dead worms; caseous material present
1279	5	176	16	2	0	1	46	Mass of live worms
1281	5	192	8	0	0	0	6	Mass of live worms
1271	5	88	48	6	0	6	26	Mass of live worms
1269	5	148	4	0	0	-	-	Dead on 4th day; no autopsy
1258	5	380	204	14	6	4	38	Small mass of live worms
1299	5	324	52	3	0	3	36	Mass of live worms
1275	5	348	18	0	0	-	-	Dead on 4th day; no autopsy
1291	5	276	4	20	0	6	84	Mass of live worms
1300	5	260	80	8	4	-	-	Dead on 4th day; no autopsy

recurrence is related somewhat to the amount and frequency of dosage, although no predictions can be made in this respect. No definite relationship exists between the recurrence of microfilariae and the height of the initial count

(Table V), nor does the height of the initial count affect the rapidity with which the microfilariae disappear (Tables I and II).

The rapidity of the reduction of microfilariae is well illustrated in Table III which gives the results obtained when treatments were administered every two hours. Within two hours after the first oral dose of 25 mg. per kilogram had been administered all animals showed a reduction of over 90 per cent in circulating microfilariae. At the end of twelve hours of treatment (at 5, 10, and 25 mg. per kilogram) many of the rats exhibited no microfilariae in the peripheral blood.

Microfilariae from cotton rats and frogs undergo contortion and spasmodic contractions when placed in solutions of the drug. The microfilariae of *Folyella dolichoptera* from southern frogs<sup>3</sup> demonstrate this effect very well when placed in dilutions of 1:100, 1:1,000, and 1:10,000 of 1-diethylcarbamyl-4-methylpiperazine hydrochloride. These microfilariae are very large embryos with a narrow whiplike anterior and a thicker posterior end. When placed in contact with solutions of the drug, the anterior end of the embryos contracts immediately into a tight coil, and violent, jerky movements occur. They then straighten out and become completely motionless within five to fifteen minutes. The same effect can be observed upon frog microfilariae in vivo during treatment. Similar effects occur with the microfilariae of *Litomosoides carinii*, although to a lesser extent.

These observations, plus the fact that adult filariae removed from cotton rats in early stages of treatment show no demonstrable damage to the embryos in utero, lead us to believe that this compound acts directly upon the microfilariae in the peripheral blood.

TABLE IV. EFFECT OF 84-L ON THE MICROFILARIAE OF *DIROFILARIA IMMITIS*

DOG	DOSE (MG./KG.)	MICROFILARIAE PER 0.05 C.C. BLOOD									
		DAYS DURING TREATMENT									
		1	4	7	14	21	28	35	42	49	
100	25 I.P., b.i.d.	896	41	30	1	6	3	-	-	-	-
221	25 Orally, t.i.d.	378	-	19	1	2	1	0	4	2	
222	25 Orally, t.i.d.	735	-	29	0	0	2	1	1	2	
260	25 Orally, t.i.d.	734	316	39	50	12	27	20	18	-	
262	25 Orally, t.i.d.	235	265	-	32	14	4	-	-	-	

In dogs infected with *Dirofilaria immitis*, 1-diethylcarbamyl-4-methylpiperazine hydrochloride produces marked reductions in the number of microfilariae during treatment. This effect is most striking in animals with high initial counts (Table IV). The rate of disappearance in dogs is not so rapid as in cotton rats, but within one or two weeks, more than a 90 per cent reduction generally occurs.

*Effect of 1-Diethylcarbamyl-4-Methylpiperazine Hydrochloride Against Adult Filariae in Cotton Rats.*—The effects of treatment with this drug on adult filariae in the cotton rat are not demonstrable so quickly as in the case of the microfilariae. It was apparent from the beginning of this work that demonstrable lethal effects on the adult worms involved a number of variables. The

most important of these have been found to be (1) the amount of drug given, (2) the frequency of dosage, and (3) the number of days elapsing from cessation of treatment to autopsy.

Autopsies performed on untreated rats revealed a small number of dead worms in nine of sixty-five (13.9 per cent). No evidence of massive deaths, adhesions, or large deposits of exudate were found in any of these animals, however, and the number of dead worms formed but a small percentage of the total number present.

TABLE V. COMPARISON OF TREATMENT THREE TIMES DAILY FOR FOURTEEN DAYS AND FOR THIRTY DAYS (81-L, 25 Mg./Kg.; COTTON RATS)

RAT	MICROFILARIAE PER 100 FIELDS								AUTOPSY RECORD (42ND AND 52ND DAYS)
	DAYS								
	1	7	14	21	28	35	42	51	
<i>Treatment Three Times Daily for Fourteen Days</i>									
904	18	0	0	6	1	14	18	Small mass of live worms; 4 dead worms	
925	36	0	0	6	20	0	1	15 live worms; small clump of dead and disintegrating worms	
913	40	0	0	6	34	56	112	Large mass of live worms; 3 dead worms	
895	72	0	0	0	0	0	0	Approximately 30 worms, half of them dead and disintegrating	
930	72	0	0	8	8	16	64	Small mass of live worms; 7 dead worms	
921	96	1	0	2	0	2	10	Mass of live worms; none dead	
905	116	0	1	6	12	42	56	Mass of live worms; none dead	
637	156	0	0	1	0	0	0	Mass of live worms; small clump of dead worms	
931	160	0	0	4	8	60	10	Large mass of live worms; approximately 20 dead worms	
940	168	0	0	14	8	0	0	12 to 15 live worms; 5 to 8 dead worms	
775	224	1	0	1	4	0	4	Small mass of live worms; none dead	
936	288	6	1	30	38	48	112	Mass of live worms; several dead worms	
924	292	0	0	40	140	172	60	Mass of live worms; none dead	
<i>Treatment Three Times Daily for Thirty Days</i>									
1079	52	0	0	2	3	12	76	Mass of live worms; several clumps of dead worms	
1050	72	0	0	0	0	0	6	Large mass of worms; majority dead	
1051	96	1	0	0	0	0	0	Mass of live worms; small clump of dead worms	
1089	112	2	2	1	1	0	48	Mass of live worms; small clump of dead worms	
1074	124	6	0	0	0	0	0	Extensive adhesions; 1 live worm; 1 dead worm	
1057	140	16	3	0	3	37	308	Large mass of live worms; none dead	
1069	160	2	0	1	0	8	40	Mass of live worms; none dead	
1054	208	0	1	1	3	10	44	Extensive adhesions; clump of dead worms; several living worms	
1078	260	3	0	0	0	3	110	Mass of worms; approximately half of them dead	
1093	264	2	2	0	0	4	24	Extensive adhesions; several live worms	
1070	324	0	0	0	1	1	60	Several live and several dead worms	
1094	440	0	0	0	0	4	96	Mass of live worms; one small clump of dead worms	

Of 150 rats treated with 1-diethylcarbamy1-4-methylpiperazine hydrochloride, with doses of 10 mg. per kilogram or more, twice or more daily, 72 per cent showed either some or all adults dead at autopsy or no adult worms.

Table VIII presents in summary form the results of autopsies on 212 rats treated with doses of from 3.13 to 100 mg. kilogram twice or more daily for from two to four weeks. Both oral and intraperitoneal treatments have been included since there is no measurable difference in the effect produced by either of these routes of administration. In general, animals treated with less than 10 mg. per kilogram did not exhibit as many dead worms at autopsy as did those treated with 10 mg. or more per kilogram.

The frequency of dosage also influenced the rapidity with which adult worms were killed. In Table III, for example, dead worms were found on the twelfth day in many rats after dosage was administered every two hours for two days. Dosage three times daily has given more consistent results than dosage twice daily.

Dead worms have been found in animals treated for from two to four weeks, and, as illustrated in Table V, there was no real difference in the condition of the adult worms following these treatment periods. A more striking difference occurred in animals subjected to autopsy at different periods after cessation of treatment. This is illustrated in Table IX and by the comparison of Tables V and X. Of the thirty-eight rats for which data are given in Table X, only three (7.9 per cent) showed all living worms at autopsy. These rats were treated orally three times daily for thirty days with 25 mg. per kilogram, and were then held seventy-seven days before autopsy. All these animals showed relatively high initial microfilarial counts, but in many of them no worms were found at autopsy.

More frequently than not, in animals that showed a rapid and sustained reduction in microfilariae during and after treatment, most of the adult worms were dead or none were found at autopsy. This is illustrated in Tables VI and X. Exceptions did occur, however, as demonstrated in Table VII.

*Effect of 1-Diethylcarbamy1-4-Methylpiperazine Hydrochloride Against Adult Filariæ in Dogs.*—Twenty-five filaria-infected dogs have been treated thus far with this compound or related derivatives. The results are encouraging in that the doses necessary to produce an effect against the microfilariae, and in some cases against the macrofilariae, do not produce signs of severe toxicity. Moreover, oral treatment is effective.

In Table XI, data are given from six dogs treated with intraperitoneal doses. In two of the dogs (Dogs 94 and 108) no adult worms were found in the heart, pulmonary artery, or lung at autopsy. Both of these animals were killed fifty days after the last treatment, and no microfilariae were found in the blood at the time of autopsy. It will be noted, however, that these animals exhibited relatively low initial embryo counts. It has been shown previously<sup>2</sup> that in many untreated dogs with low initial microfilarial counts the adult worms cannot be found at autopsy in any part of the body. A more certain criterion of cure, therefore, is the presence of dead worms in the terminal branches of the pulmonary artery.

TABLE VI. TREATED ANIMALS IN WHICH DEATH OR ABSENCE OF ADULT WORMS AT AUTOPSY WAS ASSOCIATED WITH CONSISTENTLY LOW EMBRYO COUNTS DURING AND AFTER TREATMENT

RAT	S <sup>4</sup> -L (MG./KG.)	NUMBER OF DAYS TREATED	MICROFILARIAE PER 100 FIELDS												DAY OF AUTOPSY	AUTOPSY RECORD
			DAYS													
			1	7	14	21	28	35	42	49	58	107				
481	3 I.P., b.i.d.	16	68	0	2	1	4	4						43	No worms found	
488	3 I.P., b.i.d.	16	132	1	1	0	0	1	0					43	2 clumps of dead worms; none living	
543	3 I.P., b.i.d.	30	60	0	0	0	0							31	Several dead worms; none living	
629	3 I.P., b.i.d.	32	128	3	0	0	0	0	0	0	0	0	0	70	No worms found	
617	25 I.P., b.i.d.	28	108	0	0	0	0	1	0	8	1	0	0	58	2 live worms; 2 dead worms	
599	25 I.P., b.i.d.	28	64	0	0	0	0	0	0	4	1	1	1	58	4 live worms; 6 clumps of dead worms	
593	25 I.P., b.i.d.	30	22	0	0	0	0	0	2	1				44	3 dead worms; none living	
624	25 I.P., b.i.d.	30	88	0	2	1	0	3	4					44	2 dead worms; none living	
1050	25 I.P., t.i.d.	30	72	0	0	0	0	0	6					52	Large clump of dead worms; several living worms	
1074	25 I.P., t.i.d.	30	124	0	0	0	0	0	0					52	Extensive adhesions; 1 live worm; 1 dead	
547	25 Orally, b.i.d.	30	26	0	0	0	0							33	Several dead worms; none living	
925	25 Orally, t.i.d.	14	36	0	0	6	20	0	1					42	15 live worms; several small clumps of dead worms	
895	25 Orally, t.i.d.	14	72	0	0	0	0	0	0	0				42	25 to 30 worms; half of them dead	
940	25 Orally, t.i.d.	14	168	0	0	14	8	0	0	0				42	12 to 15 live worms; 5 to 8 dead worms	
637	25 Orally, t.i.d.	14	156	0	0	1	0	0	0	0				42	Mass of live worms; several clumps of dead worms	
1325	25 Orally, t.i.d.	30	140	0	0							0		107	2 small clumps of dead worms; none living	
1402	25 Orally, t.i.d.	30	330	0	0							0		107	No worms found	
1406	25 Orally, t.i.d.	30	820	0	0							0		107	No worms found	
1409	25 Orally, t.i.d.	30	130	0	0							0		107	No worms found	
1418	25 Orally, t.i.d.	30	124	0	0							0		107	No worms found	
1425	25 Orally, t.i.d.	30	84	0	0							0		107	No worms found	
1511	25 Orally, t.i.d.	30	160	0	0							0		107	No worms found	
1540	25 Orally, t.i.d.	30	145	1								0		107	No worms found	
1544	25 Orally, t.i.d.	30	86	0	0							6		107	No worms found	
1553	25 Orally, t.i.d.	30	96	0	0							0		107	No worms found	
1368	25 Orally, t.i.d.	30	56	0	0							0		107	Two small clumps of dead worms; none living	
1403	25 Orally, t.i.d.	30	28	0	0							0		107	No worms found	
1421	25 Orally, t.i.d.	30	210	0	0							0		107	No worms found	
1414	25 Orally, t.i.d.	30	14	6	6							0		107	No worms found	
1453	25 Orally, t.i.d.	30	36	0	0							3		107	2 live worms; small clump of dead worms	
634	50 Orally, b.i.d.	28	56	0	0	0	0	1	4	8	8			58	1 live worm; clump of dead worms	
611	50 Orally, b.i.d.	28	44	0	0	0	0	0	0	0	0			58	No worms found	
569	50 Orally, b.i.d.	30	16	0	1	0	0							33	No worms found	
623	50 Orally, b.i.d.	28	52	0	0	0	0	0	0	0	0			30	No worms found	
552	100 I.P., b.i.d.	30	36	0	0	0	0							33	Several dead worms; none living	



TABLE VII. TREATED ANIMALS IN WHICH LOW EMBRYO COUNTS AT AUTOPSY DID NOT SIGNIFY DEATH OF THE MAJORITY OF ADULT WORMS

RAT	S4-L (MG./KG.)	NUMBER OF DAYS TREATED	MICROFILARIAE PER 100 FIELDS										DAY OF AUTOPSY	AUTOPSY RECORD
			DAYS											
			1	7	14	21	28	35	42	49	58			
484	3 I.P., b.i.d.	16	224	8	6	7	0	0				36	Several live worms	
486	3 I.P., b.i.d.	16	236	2	0	0	1	0				36	Several live worms	
487	3 I.P., b.i.d.	16	204	6	4	16	24	64			0	58	Several live worms	
480	6¼ Orally, b.i.d.	15	46	10	1	1	2	0			0	49	Mass of live worms	
908	10 Orally, t.i.d.	14	408	1	0	6	24	10	8			42	Mass of live worms; 4 dead worms	
917	10 Orally, t.i.d.	14	1,260	0	1	0	6	1	0			42	Mass of live worms; small clump of dead worms	
548	25 I.P., b.i.d.	30	184	0	0	0	0	0				35	Mass of live worms	
921	25 Orally, t.i.d.	14	96	1	0	0	2	0	2	10		42	All worms alive	
775	25 Orally, t.i.d.	14	224	1	0	0	1	4	0	4		42	All worms alive	
1051	25 Orally, t.i.d.	30	96	1	0	0	0	0	0		0	52	Large mass of live worms; 1 small clump of dead worms	
621	50 Orally, b.i.d.	28	608	0	0	0	0	0	0	0		42	Large mass of live worms	
555	50 Orally, b.i.d.	30	100	0	1	0	0	0	0			35	Mass of live worms	

TABLE VIII. EFFECT OF DIFFERENT DOSES OF S-I-L ON ADULT FILARIAE IN COTTON RATS, AS COMPARED WITH NONTREATED CONTROLS

DOSE* (MG./KG.)	NUMBER OF RATS	CONDITION OF ADULT WORMS AT AUTOPSY			
		ALL ALIVE (%)	LESS THAN 50 PER CENT DEAD (%)	MORE THAN 50 PER CENT DEAD (%)	ALL DEAD OR NONE FOUND (%)
3.13	31	63.2	14.7	8.8	13.3
5 and 6¼	28	78.7	14.3	7.0	0.0
10 and 12½	27	40.7	48.1	7.4	3.8
25	101	21.0	42.3	15.3	18.4
50 and 100	19	31.5	15.7	26.3	26.5
Total	212	40.5	33.9	13.2	12.4
Nontreated controls	65	86.1	13.9	0.0	0.0

\*Including treatments twice or more daily for from two to four weeks. Autopsies were performed at varying periods of time after cessation of treatment.

TABLE IX. EFFECT OF S-I-L AGAINST FILARIASIS IN COTTON RATS (AUTOPSIES AT FOUR-, SIX-, AND EIGHT-WEEK PERIODS; TREATMENT FOR FOUR WEEKS)

RAT	DOSE (MG./KG., B.I.D.)	MICROFILARIAE PER 100 FIELDS										WEEK OF AUTOPSY	AUTOPSY RECORD
		WK.											
		0	1	2	3	4	5	6	7	8			
623	50 Orally	52	0	0	0	0						4	No worms found
601	25 I.P.	34	1	0	1	0						4	2 live worms; clump of dead and deterio- rated worms
600	25 I.P.	64	0	3	1	0						4	Mass of worms, ap- proximately 50% dead
602	25 I.P.	40	2	1	0							3	Several live and sev- era dead worms
606	50 Orally	52	1	0	0	0						4	Several live and sev- eral dead worms
607	50 Orally	108	2	0	0	0						4	Several live and sev- eral dead worms
615	25 I.P.	22	1	2	1	4						4	Mass of live worms
619	50 Orally	288	1	1	0	0						4	Mass of live worms
593	25 I.P.	22	0	0	0	0	2	1				6	3 dead worms; none living
624	25 I.P.	88	0	2	1	0	3	4				6	2 dead worms; none living
592	25 I.P.	52	0	1	0	0	0	14				6	1 live worm; none dead
620	50 Orally	104	0	1	3	0	4	10				6	6 live worms
621	50 Orally	608	0	0	0	0	0	0				6	Mass of live worms
626	50 Orally	224	0	0	0	1	4	28				6	Mass of live worms
611	50 Orally	44	0	0	0	0	0	0	0	0		8	No worms found
634	50 Orally	56	0	0	0	0	1	4	8	8		8	1 live worm; clump of dead worms
616	25 I.P.	152	1	1	1	0	5	4	3	72		8	2 live worms; mass of dead worms
599	25 I.P.	64	0	0	0	0	0	4	1	1		8	4 live worms; mass of dead worms
618	50 Orally	68	0	0	0	0	6	6	14			8	1 live worm; several dead worms
590	50 Orally	52	1	2	0	0	4	12	28	18		8	Several live worms; several dead worms
617	25 I.P.	108	0	0	0	1	0	8	1	0		8	2 live worms; 2 dead worms
608	50 Orally	100	0	1	1	0	32	22	72	68		8	Several live worms; none dead

## K. EFFECT OF S1-L AGAINST FILARIASIS IN COTTON RATS (ORAL TREATMENT, 25 MG. PER KILOGRAM, THREE TIMES DAILY FOR THIRTY DAYS)

MICROFILARIAE PER 100 FIELDS			AUTOPSY RECORD (DAY 107, 77 DAYS AFTER LAST TREATMENT)
DAYS			
1	15	107	
820	0	0	No worms found
160	0	0	No worms found
145	1	0	No worms found
86	0	6	No worms found
96	0	0	No worms found
28	0	0	No worms found
210	0	0	No worms found
14	0	0	No worms found
330	0	0	No live worms found; probable remnants of dead worms
130	0	0	No live worms found; probable remnants of dead worms
124	0	0	No live worms found; probable remnants of dead worms
84	0	0	No live worms found; some adhesions
140	0	0	No live worms found; two small clumps of dead worms
56	0	0	No live worms found; two small clumps of dead worms
170	1	24	1 live worm; 1 dead worm; probable remnants of other dead worms
36	0	3	2 live worms; small clump of dead worms
200	1	36	5 or 6 live worms; small clump of dead worms
500	1	120	Small mass of live worms; two small clumps of dead worms
340	0	260	Small mass of live worms; 2 or 3 dead worms
370	1	48	Mass of live worms; two small clumps of dead worms
280	0	72	Large mass of live worms; two small clumps of dead worms
400	0	310	Several live worms; several dead and disintegrated worms
320	0	440	Large mass of live worms; one small clump of dead worms
410	0	56	Large mass of live worms; small clump of dead worms
260	0	220	Large mass of live worms; small clump of dead worms
200	2	280	Large mass of live worms; three clumps of dead worms
180	0	14	7 live worms; 2 or 3 dead worms
280	0	400	Mass of live worms; two clumps of dead worms
440	1	96	Small mass of live worms; small clump of dead worms
120	0	72	Small mass of live worms; two small clumps of dead worms
900	0	480	Mass of live worms; two large clumps of dead worms
540	0	112	Mass of live worms; small clump of dead worms
740	0	390	Large mass of live worms; none dead
36	0	0	3 live worms; none dead
440	0	92	2 live worms; none dead
96	1	112	3 live worms; 1 dead worm
560	0	68	2 live worms; 2 dead worms
320	0	48	Small mass of live worms; small clump of dead worms

In three dogs (Table XI, Dogs 101, 95, and 71) no worms were found in the heart at autopsy, but living worms were recovered from various portions of the pulmonary artery, some in the terminal branches. The remaining dog (Table XI, Dog. 100) was killed relatively soon after cessation of treatment because of a severe case of distemper. Live adults were found in the heart of this animal, but others occurred in the pulmonary artery at various levels.

Data from ten dogs treated orally with 84-L are given in Table XII. In two of these dogs (Dogs 191 and 193) no adult worms were found at autopsy. Marked reductions in microfilariae occurred in all animals with high initial counts, and in three dogs (Dogs 222, 260, and 261) many dead worms were found in the terminal lobes of the lungs. Extensive areas of infarction surrounded the plugged vessels. Upon section,\* worms in various stages of disintegration were found.

Dog 225 died after two doses of the drug had been administered. This animal had a very high initial microfilarial count, was severely emaciated, and coughed persistently before and during treatment. Within a short time after the first dose was given it was noted that the animal breathed with difficulty and was in considerable distress. It was found dead in the cage shortly after the second dose had been given. At autopsy a large mass of worms was found in the pulmonary artery near the heart, and these completely plugged the vessel. Since this is the only instance in which this condition occurred we are not convinced that it was due to the administration of the drug. It is quite possible, however, that a simultaneous migration of this large mass of worms from the right ventricle to the pulmonary artery, caused by the presence of the drug in the circulatory system, might have caused the death of the animal.

In addition to the effects produced by the drug on the filariae in dogs, it is of interest that most dogs placed under treatment improved markedly in physical appearance. Most of the dogs when received were emaciated, sickly, and unkempt. Good care and adequate food probably accounted for some of their subsequent improvement, but even in dogs with high initial microfilarial counts, a marked gain in weight and disappearance of symptoms which might be attributed to the presence of *Dirofilaria* took place during and after treatment.

As yet, too few dogs have been used to determine an optimum treatment schedule, but it seems apparent that frequent administration of the drug for several weeks is desirable. Toxicity in dogs with therapeutic doses has thus far been limited to occasional transient nausea.

#### DISCUSSION

The effect produced against microfilariae in cotton rats and dogs by 1-diethylearbamyl-4-methylpiperazine hydrochloride is certainly more rapid than that reported for any other known compound. Moreover, to our knowledge, this is the first compound shown to produce this effect in experimental animals when administered by the oral route. No data have been obtained thus far with regard to the rate of absorption and excretion of this com-

\*Sections prepared and studied by Dr. Frederick Dessau.

pound, but indirect evidence denotes that it is absorbed rapidly when given either orally or parenterally. The better effects produced by frequent dosage indicate that it either is excreted rapidly or is degraded in the body.

Studies are now in progress regarding the fate of the embryos once they are removed from the peripheral circulation.

The failure of this compound to kill all adult worms in all animals treated may be due in part to individual variations in the absorption of the drug. Furthermore, the state of maturity of adult worms, or the number of worms present in the pleural cavity, may influence the effectiveness of treatment. There is no question concerning the more rapid lethal effect of parenteral doses of several antimony derivatives and cyanine dyes<sup>4-7</sup> against adult worms in cotton rats. We believe it important, however, that for the first time an organic, non-metallic compound has been found which produces a very marked effect against filariasis in experimental animals when given by mouth.

It should be pointed out that many different dosage regimes have been included in the 212 treated rats for which summary data are given in Table VIII. The best results, as shown in Table X, have been obtained by treating three times daily for thirty days, and then holding the animals for two months before autopsy. In many of the rats treated by this method, no adult worms were found at autopsy, in spite of the fact that high microfilarial counts were present before treatment was initiated. In other rats in this series, varying numbers of dead adult worms were found at autopsy, and in three rats all adult worms were alive. These data, plus the fact that microfilariae sometimes do and sometimes do not recur in the peripheral blood after cessation of treatment demonstrate that a number of unknown variables probably influence the effectiveness of treatment.

The mode of action of this compound against micro- or macrofilariae in the cotton rat is not known. Worms have been found in various stages of decay and disintegration within the pleural cavity after treatment. Worms recovered shortly after death occurred showed no movement when placed in physiologic saline, but no external or internal changes in the appearance of these worms could be noted. Living or dead embryos occurred within the uteri. In other cases, dead worms were found enveloped in caseous, purulent material such as has been described by Culbertson and Rose<sup>4</sup> following treatment with Neostibosan and Neostam. In very late stages of decay the worms occurred in tight clumps, were usually embedded in a firm, yellowish mass, and were fragmented. In the many cases where no worms were found, the chest cavity was clean and no evidence of fragments of dead worms was found. We have assumed that the dead worm tissue is eventually absorbed.

In some cases where large masses of dead worms were found, extensive adhesions of the heart and lung to the walls of the pleural cavity occurred.

The data obtained thus far from filaria-infected dogs treated with 1-diethylcarbamyl-4-methylpiperazine hydrochloride suggest that this drug may prove useful in the treatment of this infection. As in the case of the cotton rats, individual variations occurred in the susceptibility of the worms to treatment. Emphasis has not been placed upon treated cases in which no adult worms

TABLE XI. RESULTS OF TREATMENT OF DIROFILARIA-INFECTED DOGS WITH INTRAPERITONEAL INJECTIONS OF 84-L

DOG	DOSE (MG./KG.)	MICROFILARIAE PER 0.05 C.C.										MICRO- FILARIAE (LAST DAY BE- FORE AUTOPSY)	AUTOPSY (DAYS AFTER LAST TREAT- MENT)	AUTOPSY RECORD
		WEEK												
		0	1	2	3	4	5	6	7	8	9			
100	25 I.P., b.i.d., for 14 days	896	30	1	6	3						3	16	Killed because of severe dis- temper; 36 live worms; several in heart, others in pulmonary artery at var- ious levels
101*	50 I.P., b.i.d., for 13 days	354	16	1	13	11	21	62	67			67	41	No worms in heart; 15 live worms in pulmonary artery near heart; 10 live worms in pulmonary vessels in center of lung
95*	50 I.P., b.i.d., for 13 days	130	12	63								63	2	3 live worms in pulmonary artery; 1 live worm in lung, nearly at terminus of blood vessel
94	50 I.P., b.i.d., for 9 days	8	0	2	0	0	3	1		0		0	50	No worms found
	100 I.P., b.i.d., for 2 days													
108	50 I.P., b.i.d., for 9 days	28	10	18	1	5				0		0	50	No worms found
	100 I.P., b.i.d., for 2 days													
71	50 I.P., b.i.d., for 13 days	22	5	19	7	11	7	18		38		38	50	No worms in heart; small clump of live worms (3 or 4) in terminal pulmonary vessel

\*Treated several weeks previously with 25 mg. per kilogram b.i.d. for fifteen days. Microfilariae count reduced, but relapsed upon cessa-  
tion of treatment.

\*Treated several weeks previously with 25 mg. per kilogram b.i.d. for fifteen days. Microfilariae count reduced, but relapsed upon cessa-  
tion of treatment.

TABLE VII. RESULTS OF TREATMENT OF MICROFILARIA-INFECTED RATS WITH CHEAT DIONE OF C-11

DOG	DOSE (MG./KG.)	MICROFILARIAE PER 0.05 C.C.														MICRO- FILARIAE (LAST DAY BEFORE AUTOPSY)	AUTOPSY (DAYS AFTER LAST TREAT- MENT)	AUTOPSY RECORD	
		WEEK																	
		0	1	2	3	4	5	6	7	8	9	10	No worms found						
191	25 Orally, t.i.d., for 23 days	1	0	0	3	0	0	0	1	0	0				49	No worms found	No worms in heart; one pair of live worms in pulmonary artery		
193	25 Orally, t.i.d., for 23 days	18	2	0	0	1	1	1	2	1	1				49	No worms found	No worms in heart; 15 living worms in pulmonary artery and in lung		
194	25 Orally, t.i.d., for 23 days	7	22	5	2	0	2	9	3	2	0				1	No worms found	No worms in heart; 2 live worms in pulmonary artery; 4 "cysts" in terminal lobe of lung; upon section these were found to contain worms, with various stages of organization of the thrombus; small hemorrhagic infarcts present		
221	25 Orally, t.i.d., for 64 days	378	19	1	2	1	0	4	2	1	0				17	No worms found	Animal died in afternoon of first treatment day; large mass of live worms in heart and plugged in pulmonary artery; labored breathing before and during treatment		
222	25 Orally, t.i.d., for 64 days	735	29	0	0	2	1	1	2	2	1	1				30	No worms found	10 live worms in heart; 10 live worms in pulmonary artery; 2 live worms in lung; lungs show extensive infarction; many worms found in terminal arteries with thrombus formation	
225	25 Orally, 2 doses	1,613															16	No worms found	No worms in heart; 9 live worms in pulmonary artery; no macroscopic lesions in lungs
260	25 Orally, t.i.d., for 60 days	734	39	50	12	14	4	27	20	18	20	11	2	8	172	30	No worms found	8 live worms in heart; none found in lung; no lesions found in lung	
262	25 Orally, t.i.d., for 58 days	235	265	32	14	4	26	16	5	11	134	50	3	4	3 worms in heart; 1 live worm in pulmonary artery; massive hemorrhage and infarction in lower quadrant of lower right lobe of lung; several hard cystlike structures containing remnants of dead worms; living and dead embryos within these remnants				
259*	35 Orally, t.i.d., for 27 days	232	17	14	7	3	3	43	4										
261*	50 Orally, b.i.d., for 27 days	209	58	124	88	75	43												

\*Treated several weeks previously with 30 mg. per kilogram of compound 180-C, orally, t.i.d. for forty-seven days. No sharp reduction in microfilariae occurred.

were found at autopsy, since it is well known<sup>2</sup> that adult worms cannot always be found in untreated dogs which show microfilariae in the blood stream, particularly when the microfilariae are few in number. It is believed important, however, that in three dogs with relatively high initial microfilaria counts large number of dead adult worms were found in the terminal blood vessels of the lung following treatment. These dogs showed marked physical improvement during the course of their treatment; all gained weight and the microfilariae had been reduced rapidly in number. A large series of cases has now been put under observation in order to determine an optimum treatment schedule. It is emphasized again that no disturbing toxic symptoms have occurred during treatment, even though the drug was administered in some cases three times daily for two months. The one death which occurred after two doses had been administered was that of a dog with a very heavy worm burden, and this dog had been in obvious distress several days before treatment was started.

The use of piperazines therapeutically in man is not new, although the use to which they have been put previously has largely been abandoned. Hanzlik<sup>3</sup> gives a summary of the literature on the use of piperazine hexahydrate and several similar compounds as urate solvents. Doses as high as 6 Gm. of piperazine hexahydrate have been administered to human subjects without undesirable effects. Other investigators mentioned by Hanzlik, however, report that large doses produce headache, clonic spasms of the extremities, muscular prostration, incoordination, tremor, malaise, and nausea. Piperazine hexahydrate has been reported by several investigators<sup>4</sup> to be rapidly absorbed and excreted by human patients.

#### SUMMARY

Data are presented which show the effects produced by 1-diethylearbamyl-4-methylpiperazine hydrochloride on natural infections with filariae in cotton rats and dogs. An immediate and sustained reduction in microfilariae is produced in cotton rats following oral or parenteral administration of the drug. The effect produced against microfilariae in dogs is less rapid but is substantially the same.

Various factors which influence the effects produced on the adult worms in cotton rats are discussed. The maximum number of adult worms are killed when treatment is administered at frequent daily intervals for a period of three weeks or longer, with doses of 10 mg. or more per kilogram.

It is demonstrated that in some *Dirofilaria*-infected dogs treated with the compound the worms were not present in the heart, but were located in terminal branches of the pulmonary artery; and in three dogs, many worms were dead and surrounded by areas of hemorrhage and infarction.

#### REFERENCES

1. Hewitt, R., White, E., Wallace, W. S., Stewart, H. W., Kushner, S., and Subbarow, Y.: Experimental Chemotherapy of Filariasis. II. Effect of Piperazine Derivatives Against Naturally Acquired Filarial Infections in Cotton Rats and Dogs, *J. LAB. & CLIN. MED.* 32: 1304, 1947.
2. Hewitt, R., Wallace, W., White, E., and Subbarow, Y.: Experimental Chemotherapy of Filariasis. I. Experimental Methods for Testing Drugs Against Naturally Acquired Filarial Infections in Cotton Rats and Dogs, *J. LAB. & CLIN. MED.* 32: 1293, 1947.



3. Wehr, E., and Causey, O.: Two New Nematodes (Filaroidea: Dipetalonematidae) From *Rana Sphenoccephala*, *Am. J. Hyg.* 30: 65-68, 1939.
4. Culbertson, J., and Rose, H.: Chemotherapy of Filariasis in the Cotton Rat by Administration of Neostam and Neostibosan, *J. Pharmacol. & Exper. Therap.* 81: 181-196, 1944.
5. Culbertson, J., and Price, E.: Chemotherapy of Filariasis (*Litomosoides carinii*) in the Cotton Rat by the Administration of Stibanose (Solustibosan), *J. Pharmacol. & Exper. Therap.* 87: 181-184, 1946.
6. Welch, A., Peters, L., Bueding, E., Valk, A., Jr., and Higashi, A.: A New Class of Antifilarial Compounds, *Science* 105: 486-488, 1947.
7. Cranston, E., Cuckler, A., Litchfield, J., Jr., Brey, T., Wright, H., and Bieter, R.: Chemotherapeutic Activity of Cyanines and Related Compounds in the Cotton Rat, *Federation Proc.* 6: 318, 1947.
8. Hanzlik, P.: Piperazin and Other Organic Urate Solvents, *J. LAB. & CLIN. MED.* 2: 308-327, 1917.

## STUDIES IN ERYTHROBLASTOSIS FETALIS

### I. ACTIVATION OF THE INCOMPLETE RH ANTIBODY BY THE BLOOD SERUM OF FULL-TERM AND PREMATURE NEWBORN INFANTS

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THE failure to demonstrate Rh antibodies in the majority of sera obtained from mothers who have given birth to erythroblastotic children constituted, for a while, one of the major difficulties in accepting the theory of isoimmunization advanced by Levine and co-workers as the cause of erythroblastosis. The discovery of a second type of antibody, variously called "incomplete antibody" by Race<sup>1</sup> and "blocking antibody" by Wiener,<sup>2</sup> removed one of the many remaining puzzling problems connected with erythroblastosis. Investigations of Diamond, Abelson, and Denton,<sup>3-5</sup> as well as of Wiener,<sup>6</sup> revealed the fact that this new type of antibody agglutinates Rh-positive cells, provided that albumin solution or undiluted serum replaces physiologic saline solution as the diluent.

In spite of quantitative differences, most adult human sera can be used as the diluent for bringing about agglutination of Rh-positive cells by the incomplete antibody. In contrast to this capacity of adult sera, Lubinsky<sup>7</sup> states that umbilical cord serum cannot be used as a diluent. Wiener,<sup>8</sup> in discussing the "bizarre behavior of congenital hemolytic disease, so that some infants do not present signs of hemolysis until several hours or days after birth," tried to find an explanation for the sudden appearance of hemolysis after birth in the following mechanism. He suggests the possibility that the so-called X-protein, described by Pedersen,<sup>9</sup> is involved in the agglutination of Rh-positive cells by the incomplete antibody, and that the late onset of hemolysis is related to the development of this X-protein which does not form until after birth or at the time of birth. Gurevitch, Polishuk, and Hermoni<sup>10</sup> just recently examined umbilical cord serum and the serum of infants up to six months of age and found them devoid of this enhancing property. From this observation they came to the conclusion that there is no evidence for the presumed role of a so-called serum factor or X-protein in the pathogenesis of hemolytic disease of the newborn. Our investigations deal with the characteristics of serum in prenatal and postnatal life with regard to the activation of the incomplete Rh antibody.

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\*The term blocking antibody is considered now to be incorrect. Several other terms have been suggested, such as univalent or glutinin. The need for a generally acceptable term is apparent.

## EXPERIMENTAL

Several sera containing Rh antibodies of the incomplete variety, obtained from women with erythroblastotic children, as well as a commercially prepared serum\* containing potent antibodies of the same type, were at our disposal. In order to determine the activating capacity of the serum of normal newborn infants, the following experiment was performed:

Decreasing dilutions of serum (volume, 0.1 c.c.) of an Rh-negative patient, Mrs. B., who was delivered of an erythroblastotic child, were mixed with 0.1 c.c. of a 3 per cent suspension of Rh-positive blood cells belonging to Group O. The following diluents for both the serum dilutions and the cell suspensions were used: Column a, physiologic saline solution; Columns b, c, and d, normal adult sera belonging to Groups O, A, and B, respectively; Columns e, f, and g, three normal umbilical cord sera. The mixtures were kept in a water bath for one hour at 37° C. and then spun down at a medium speed for two minutes. The results of this experiment are seen in Table I.

TABLE I. AGGLUTINATION OF RH-POSITIVE CELLS OF GROUP O BY INCOMPLETE RH ANTIBODIES, ACTIVATED BY HUMAN ADULT SERUM AND NORMAL CORD SERUM

ANTI-RH SERUM (MRS. B.)	a	b	c	d	e	f	g
	DILUENT						
	SALINE (0.9 %)	HUMAN ADULT SERUM			HUMAN UMBILICAL CORD SERUM		
		GROUP O	GROUP A	GROUP B	DEG.	SIL.	WEI.
1:5	-	+++	+++	++++	+++	+	+++
1:10	-	++++	++++	++++	+	±	+++
1:20	-	++++	+++	+++	+	-	+++
1:40	-	++++	++	+++	±	-	++
1:80	-	+++	++	++	-	-	+
1:160	-	++	+	++	-	-	+
1:320	-	+	±	+	-	-	-
0	-	-	-	-	-	-	-

-, No agglutination; ±, faint agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination; +++, very strong agglutination.

Table I shows that the serum of Mrs. B. failed to agglutinate Rh-positive cells when saline solution was used as a diluent. If, however, normal adult serum was used, Rh-positive cells were agglutinated by Mrs. B.'s serum up to a dilution of 320. Umbilical cord sera, on the other hand, exhibited a remarkably different picture. Serum Wei. almost equalled adult sera in its ability to produce agglutination. In contrast, serum Sil. was considerably weaker in its activating capacity, and serum DeG. fell somewhere in between the two extremes. It should be mentioned that these three normal umbilical cord sera were selected at random.

Differences in the capacity of cord sera to activate the incomplete Rh antibody of various anti-Rh sera become evident from the following experiment as shown in Table II. For this experiment, serum of Mrs. Mar. (Part I), who had given birth to an erythroblastotic child, was compared with a commercial anti-Rh serum (Part II) which we assume was obtained by arti-

\*This serum was purchased from Dade County Laboratories, Miami, Fla. Several different batches were used, the first one being of considerably higher potency than the rest.

ficial immunization of Rh-negative volunteers with Rh-positive cells. The experiment was carried out in the following way:

Decreasing amounts of Rh antisera (volume, 0.1 c.c.) were mixed with 0.1 c.c. of a 3 per cent suspension of Rh-positive Group O cells. As diluents for both the Rh serum dilutions and the cell suspensions the following were used: Column a, physiologic saline solution; Columns b, c, and d, normal adult sera selected at random; Columns e, f, and g, normal cord sera selected at random. The mixtures were kept in a water bath for one hour at 37° C. and then spun down at a medium speed for two minutes. The resulting agglutination is seen in Table II.

TABLE II. AGGLUTINATION OF RH-POSITIVE GROUP O CELLS BY TWO ANTI-RH SERA CONTAINING INCOMPLETE RH ANTIBODIES OF LOWER AND HIGHER TITER

	a	b	c	d	e	f	g
	DILUENT						
	ADULT SERUM			UMBILICAL CORD SERUM			
SALINE (0.9 %)	GROUP O	GROUP A	GROUP B	FO	MA	HU	
<i>Part I</i>							
MRS. MAR.'S ANTI-RH TEST SERUM							
1:5	-	+++	+++	+++	+	-	-
1:10	-	+++	++	++	-	-	-
1:20	-	++	+	+	-	-	-
1:40	-	+	±	+	-	-	-
0	-	-	-	-	-	-	-
<i>Part II</i>							
COM- MERCIAL ANTI-RH TEST SERUM							
1:5	±	++++	++++	++++	+++	+++	+++
1:10	-	++++	+++	++++	+++	++	+++
1:20	-	+++	+++	+++	++	++	++
1:40	-	+++	++	+++	+	+	+
0	-	-	-	-	-	-	-

-, No agglutination; ±, faint agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination; +++, very strong agglutination.

Mrs. Mar.'s serum (Part I) failed to agglutinate Rh-positive cells when normal cord serum was used as a diluent, whereas adult serum brings out the presence of an incomplete Rh antibody of about average titer.

In Part II of this experiment, incomplete Rh antibodies of rather high titer (commercial Rh test serum) were activated by both adult and cord sera, though even in this case adult serum seemed to be somewhat stronger than cord serum.

The differences in results obtained with these two types of sera probably depend upon the titer of Rh antibodies present in the respective anti-Rh sera. However, the mode of production of the Rh antibody may also be a factor. In one case there was a natural production of antibody in a mother with an

erythroblastotic child, whereas in the other case Rh antibodies were presumably produced by artificial immunization.

The question arose as to whether the serum of premature children, because of its immaturity, would be capable of activating the incomplete antibody. Cord sera of premature babies were thus tested in the following manner:

Decreasing amounts of a commercial Rh test serum (volume, 0.1 c.c.) were mixed with 0.1 c.c. of a 3 per cent suspension of Rh-positive cells belonging to Group O. As diluents for both the Rh serum and the cell suspension the following substances were used: Column a, saline solution; Columns b, c, and d, adult sera selected at random; Column e, cord serum from a 6-month-old fetus who died a few minutes after birth; Columns f and g, cord serum from two premature babies of 8 months' gestation; Columns h and i, cord serum from premature babies born one to two weeks before term; Columns j, k, and l, cord sera from full-term babies. The mixtures were kept for one hour at 37° C., shaken slightly, and then spun down at medium speed for two minutes. The resulting agglutinations are shown in Table III.

TABLE III. AGGLUTINATION OF RH-POSITIVE CELLS OF GROUP O BY HIGH TITERED INCOMPLETE RH ANTIBODY ACTIVATED BY HUMAN ADULT SERUM AND THE CORD SERUM OF PREMATURE AND FULL-TERM BABIES

COM- MERCIAL ANTI-RH TEST SERUM	a	b	c	d	e	f	g	h	i	j	k	l
	DILUENT											
	SALINE (0.9 %)	ADULT SERUM			UMBILICAL CORD SERA							
		GROUP O	GROUP A	GROUP B	OZ. (6 MO.)	GO. (8 MO.)	PU. (8 MO.)	FR. (2 WK. PRE- MATURE)	BR. (1 WK. PRE- MATURE)	KI. (FULL TERM)	CR. (FULL TERM)	RE. (FULL TERM)
1:5	+	++++	++++	++++	±	++	+	+++	+++	+++	++++	+++
1:10	-	++++	++++	++++	-	+	±	+++	++	+++	++++	+++
1:20	-	+++	++	+++	-	-	-	+	±	+++	+++	++
1:40	-	+++	++	+++	-	-	-	±	-	+	++	+
1:80	-	++	±	+	-	-	-	-	-	±	+	-
1:160	-	+	-	±	-	-	-	-	-	-	±	-
0	-	-	-	-	-	-	-	-	-	-	-	-

-, No agglutination; ±, faint agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination; +++, very strong agglutination.

The anti-Rh serum under investigation produced a very weak agglutination of Rh-positive cells when diluted in saline. In normal adult serum, however, strong agglutination of Rh-positive cells occurred, notwithstanding the differences between the individual adult sera used for that purpose. The three cord sera obtained from full-term babies also activated the incomplete Rh antibody in this Rh test serum, the strongest cord serum to the same extent as an average adult serum (see Table II, Part II). In contrast, serum of premature babies was characterized by a definite weakness in activating potency, obviously depending upon the age of the babies. Umbilical cord serum from a 6-month-old fetus acted almost like saline solution, while the cord sera of two 8-month-old fetuses were only slightly better than saline solution. The impression gained from this experiment is that cord sera from babies

born one to two weeks prematurely have a somewhat lower capacity for activating the incomplete Rh antibody than the average cord serum of full-term babies. Yet in many instances even the serum of full-term infants seemed to be completely devoid of this activating capacity when tested against maternal Rh antiserum of low or average titer, as seen in Table II. Obviously a maturation factor plays a definite role in the appearance of the characteristics responsible for the activation of the incomplete Rh antibody, as demonstrated by means of a suitable anti-Rh serum containing a potent antibody of the incomplete variety.

In the preceding experiment the maturation principle was shown to be operating during prenatal life. The following experiment was designed to show the continued maturation of this activating capacity in postnatal life. For this purpose an anti-Rh serum whose incomplete antibody was not activated by normal cord serum was used.

Three different serum specimens were collected from each of two babies: (1) cord serum; (2) serum from venous blood obtained twenty-four hours after birth; and (3) serum from venous blood obtained forty-eight hours after birth. Decreasing amounts of serum (volume, 0.1 c.c.) from Mrs. Matt., who had been delivered of an erythroblastotic child, were mixed with 0.1 c.c. of a 3 per cent cell suspension of Rh-positive cells belonging to Group O. Serum and cell suspensions were diluted as follows: Column a, in saline solution; Column b, in adult serum; Columns c, d, and e, in serum specimens 1, 2, and 3 obtained from full-term baby Vi.; Columns f, g, and h, in serum specimens 1, 2, and 3 obtained from full-term baby Ha. The tubes were kept in a water bath for one hour at 37° C. and then spun down at medium speed for two minutes. Table IV shows the results of this test.

TABLE IV. AGGLUTINATION OF RH-POSITIVE CELLS OF GROUP O BY AN ANTI-RH SERUM CONTAINING INCOMPLETE ANTIBODIES ACTIVATED BY ADULT SERUM AND NOT BY CORD SERUM OF NORMAL FULL-TERM BABIES

MRS. MATT.'S ANTI-RH SERUM	a	b	c	d	e	f	g	h
	DILUENT							
	SALINE (0.9 %)	POOLED HUMAN ADULT SERUM	SERA OF FULL-TERM NEWBORN BABIES					
			VI.			HA.		
			1	2	3	1	2	3
1:40	-	+++	-	++	++	-	-	-
1:80	-	++	-	+	+	-	-	-
1:160	-	++	-	±	±	-	-	-
1:320	-	+	-	-	-	-	-	-
0	-	-	-	-	-	-	-	-

- , No agglutination; ±, faint agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination.

The incomplete Rh antibody present in the serum of Mrs. Matt. is activated clearly by pooled adult serum. The cord serum of the first full-term baby, Vi., is lacking completely in activating power under the experimental conditions chosen. However, serum specimens obtained from the same baby twenty-four hours as well as forty-eight hours after birth reveal the presence of the activating principle, although it is not equal in strength to that of pooled adult serum.

In contrast, the serum of the second baby, Ha., is still negative forty-eight hours after birth. It should be added that in a different experiment not reported in detail in this paper, a third baby's serum failed to activate the incomplete Rh antibody twenty-four hours after birth, but did so forty-eight hours after birth. It is evident from these experiments, therefore, that in some instances the capacity of a baby's serum to activate the incomplete Rh antibody increases in strength soon after birth and is subject to individual variation.

Considering the fact that the average normal cord serum does not activate the incomplete Rh antibody to the same extent as the average adult serum, the theoretical question is raised as to whether the transfusion of erythroblastotic children with adult's blood may be, at times, contraindicated, due to the fact that the infused adult plasma might activate the incomplete antibody present in the baby. The experimental analysis of this problem presents several difficulties. One of them is that the plasma portion of whole blood taken up in citrate or in a.c.d. solutions is diluted by 75 or 120 c.c. of the crystalloid solution per 500 c.c. of whole blood, resulting in a definite dilution of the blood plasma originally present. In a few experiments it was found that citrated plasma, when compared with serum obtained from the same patient, did not activate the incomplete antibody as well as the serum itself and that the addition of as little as 1 part of saline solution to 2 parts of serum reduced the activating power of the serum remarkably.

Samples of blood obtained from erythroblastotic babies at different periods during exchange transfusions were tested to determine their activating capacities. It was anticipated that with progress of the transfusion the activating capacity of the samples would increase due to the increased percentage of adult plasma in the samples withdrawn. The results of this study in three instances are too irregular to permit the drawing of any final conclusions. Further investigations are needed to elucidate this point.

#### COMMENTS

On the whole, serum obtained from the umbilical cord of full-term infants, when compared with the average normal adult serum, is characterized by a decreased capacity to activate the incomplete Rh antibody. However, considerable variations do occur. Some adult sera are rather potent in this respect, while others are of relatively low potency. There are a few cord sera which are as strong as adult sera of low or average potency, while other cord sera are weaker than the weakest adult sera tested so far. The susceptibility of the red blood cells used also varies, inasmuch as certain cells are clumped by the incomplete antibody more readily than others. As pointed out by other investigators, agglutination of red blood cells by the incomplete Rh antibody rarely reaches the strength of agglutination frequently observed by the interaction of a strong saline Rh antibody with the corresponding red blood cells.

The serum protein, albumin, and globulin levels were determined on several adult and normal cord sera obtained from full-term babies used in these experiments. In general, the average cord sera contained about 1 Gm. per cent less

of total protein and  $\frac{1}{2}$  Gm. per cent less of both albumin and globulin than was found in the average adult serum. However, some of the cord sera which poorly activated the incomplete Rh antibody had serum albumin and globulin levels comparable to those of adult sera which activated the incomplete antibody well. Furthermore, there were no striking differences between protein fractions in the cord sera which did and did not activate the incomplete antibody. Studies of serum protein fractions by more complete methods might give further information.

The experiments reported were carried out by warming the tubes in a water bath at 37° C. This temperature, however, is not essential; room temperature and icebox temperature seem to be equally suitable. The length of incubation necessary to secure maximal agglutination also varies, and while in our experiments the blood-serum mixtures were allowed to stand for one hour, we have the impression that one-half hour is sufficient.

The time and speed of centrifugation are of great importance and determine in many instances the degree of agglutination obtained. The advantage of increased agglutination following prolonged centrifugation at higher speed is frequently outbalanced by the necessity for shaking sediments more intensely at the time of reading, resulting in the breaking up of the clumped cells.

There are certain clinical implications which present themselves as a result of observations reported in this paper. The activating power of serum is apparently dependent upon a maturation factor. Serum of a 6-month-old fetus was practically devoid of activating potency, and the serum of a 7- to 8-month-old fetus showed definitely less activating potency than the cord serum of most full-term babies. The maturation factor becomes apparent and can be easily demonstrated if certain high titered anti-Rh sera, whose incomplete antibody can be activated also by the average normal cord serum, are used. The low titered anti-Rh sera which are not activated even by the normal average cord serum are not useful for the demonstration of the maturation principle during prenatal life. It seems reasonable to entertain the possibility that a certain relationship exists between the capacity of the baby's serum to activate the incomplete antibody and the clinical picture of erythroblastosis. The development of this maturation factor might explain the fact that clinical manifestations become evident only during the second part of pregnancy. In some instances babies are born apparently normal and develop clinical manifestations shortly after birth. This might in some way be related to a rapid increase in activating potency after birth, as was found in several instances.

Because of individual variation, it is not possible to predict the actual status of the activating power of the fetus' serum at any given time. However, the assumption that the factor constantly increases in potency during the last half of pregnancy is justified. Undoubtedly, therefore, the babies are exposed both to the mother's Rh antibody passing from the maternal into their own circulation and to the development of an increased activating capacity of their own blood plasma. The level of activating properties of the serum of erythroblastotic babies examined so far was not appreciably higher than that



of the sera of normal infants of the same age. The school of investigators favoring premature delivery as a therapeutic measure in erythroblastosis fetalis might find additional support for their belief in the observations reported. However, careful clinical judgment should be applied in order to avoid adding the trials of prematurity to erythroblastosis. The induction of premature labor three to four weeks before term in cases where there are reasons to suspect an erythroblastotic baby deserves serious consideration by the obstetrician, who must ultimately make the decision after analysis of the whole problem.

The question of treating erythroblastotic babies with transfusions of adult blood deserves critical thought. Even though it is not possible as yet to prove experimentally an increase of activating potency in the baby's blood plasma following the injection of whole blood, the conclusion that adult plasma given to erythroblastotic babies might possibly increase the danger of intravascular agglutination and hemolysis seems inescapable. An exchange transfusion replacing the larger part of the baby's blood cells would therefore seem to have promise of better results than the simple transfusion of adult whole blood. Furthermore, the transfusion of blood minus plasma (washed red cells) deserves consideration, especially in the milder cases. That even relatively small amounts of adult serum mixed with newborn serum increase the activating power of that newborn's blood serum will be described in a subsequent communication.<sup>11</sup>

#### CONCLUSIONS

1. The cord sera of full-term newborn babies are compared with normal adult sera regarding their relative capacities to activate the incomplete Rh antibody and produce agglutination. The results depend upon the type of anti-Rh serum selected for this purpose. Certain anti-Rh sera containing incomplete antibodies of low or even average titer, originating from patients with erythroblastotic children, were activated by normal adult sera, but only slightly, or not at all, by normal cord sera. In contrast, other anti-Rh sera, such as those supposedly produced by immunization of volunteers and containing incomplete Rh antibodies of fairly high titer, were activated by both normal adult serum and normal cord serum. However, even in the latter instance, the average normal cord serum frequently proved to be of somewhat weaker potency than the average normal adult serum.

2. A considerable difference could be demonstrated between the cord serum of babies born prematurely and those born at term when anti-Rh sera containing incomplete Rh antibodies of high titer were used. The cord serum of a 6-month-old fetus seemed to be lacking completely in activating potency, resembling saline solution in this respect. The cord serum of babies born four to eight weeks prematurely exhibited some degree of activating potency though less than the cord serum of full-term babies. The activating power of serum, therefore, depends upon a maturation principle which is subject to considerable individual variation.

3. The maturation of the activating capacity may continue after birth. In order to demonstrate this principle, anti-Rh sera containing incomplete anti-

bodies which fail to produce agglutination with cord serum of full-term babies should be selected. Using anti-Rh serum of this type, differences in the activating power of normal cord serum and normal adult serum become readily apparent. Serum specimens from two of three normal babies taken twenty-four to forty-eight hours following delivery showed an increase in activating power when compared with their respective cord serum, although they had not reached the strength of normal average adult serum. Further studies of the maturation factor in postnatal life are necessary.

4. Considerable individual differences in the activating potency of various sera, cord as well as adult, and differences in the susceptibility of various individual blood cells to agglutination influence the titer of agglutination obtained. The theoretical implications of the reported observations with respect to the development of the clinical manifestations of erythroblastosis and the treatment of the disease by means of transfusions of whole adult blood are discussed.

#### REFERENCES

1. Race, R. R.: "Incomplete" Antibody in Human Serum, *Nature* 153: 771-772, 1944.
2. Wiener, A. S.: New Test (Blocking Test) for Rh Sensitization, *Proc. Soc. Exper. Biol. & Med.* 56: 173-176, 1944.
3. Diamond, L. K., and Abelson, N. M.: Importance of Rh Inhibitor Substance in Anti-Rh Serums, *J. Clin. Investigation* 24: 122-126, 1945.
4. Diamond, L. K., and Abelson, N. M.: Demonstration of Anti-Rh Agglutinins—Accurate and Rapid Slide Tests, *J. LAB. & CLIN. MED.* 30: 204-212, 1945.
5. Diamond, L. K., and Denton, R. L.: Rh Agglutination in Various Media With Particular Reference to the Value of Albumin, *J. LAB. & CLIN. MED.* 30: 821-830, 1945.
6. Wiener, A. S.: Conglutination Test for Rh Sensitization, *J. LAB. & CLIN. MED.* 30: 662-667, 1945.
7. Lubinsky, H. H.: Routine Rh Testing by a Simplified Conglutination Technique, *Canad. M. A. J.* 54: 285, 1946.
8. Wiener, A. S.: Rh Factors in Clinical Medicine, *J. LAB. & CLIN. MED.* 30: 957-976, 1945.
9. Pedersen, K. O.: Ultracentrifugal Studies on Serum and Serum Fractions, Uppsala, Sweden, 1945, Almquist and Wiksells.
10. Gurevitch, J., Polishuk, Z., and Hermoni, D.: The Role of a Presumed Serum Protein in the Pathogenesis of Erythroblastosis Fetalis, *Am. J. Clin. Path.* 17: 465-468, 1947.
11. Witebsky, Ernest, Rubin, Mitchell I., Engasser, Lillian M., and Blum, Livia: Studies in Erythroblastosis Fetalis. II. Investigations on the Detection of Sensitization of the Red Blood Cells of Newborn Babies With Erythroblastosis Fetalis, *J. LAB. & CLIN. MED.* 32: 1339, 1947.

## STUDIES IN ERYTHROBLASTOSIS FETALIS

### II. INVESTIGATIONS ON THE DETECTION OF SENSITIZATION OF THE RED BLOOD CELLS OF NEWBORN INFANTS WITH ERYTHROBLASTOSIS FETALIS

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THE laboratory support of the clinical diagnosis of erythroblastosis fetalis is of ever-increasing importance in order to establish the diagnosis with certainty as early as possible. This need has become more important now that exchange transfusions have been introduced as a method of treatment in severe cases during the first hours of life.

The demonstration of Rh antibodies in the mother's serum is the first indication that she may give birth to an erythroblastotic child, and an increase in this antibody titer during the last half of pregnancy strongly suggests the probability of such an event but does not prove its inevitability. Free circulating Rh antibodies are often found in the umbilical cord serum of erythroblastotic babies. Their demonstration is frequently interpreted as a sign of antibody surplus, indicating that the Rh antigens present in the baby's cells have been completely saturated with the corresponding antibodies. Nevertheless, the presence of these abnormal antibodies in the cord serum is good indication that the baby has been sensitized and might possibly constitute sufficient reason for an immediate exchange transfusion. However, many of the patients with erythroblastosis whom we have had occasion to observe recently showed either no free circulating antibodies or only antibodies of low titer in the cord serum in spite of the fact that these babies exhibited clinical signs of severe blood destruction. In the absence of demonstrable Rh antibodies in the cord serum the laboratory evidence of the baby's sensitization rests upon the demonstration of sensitization of his red blood cells.

Several investigators have described different methods of demonstrating sensitization of the babies' blood cells. Carter and Loughery<sup>1</sup> reported that they succeeded in releasing anti-Rh agglutinins from their attachment to the blood cells of erythroblastotic babies by heating the cell suspensions in a water bath at 56° C. To demonstrate this cell sensitization, Coombs, Mourant, and Race<sup>2-4</sup> use an antihuman globulin rabbit serum which induces the agglutination of erythroblastotic babies' cells but not of normal blood cells. Hill and Haberman<sup>5</sup> have further demonstrated the usefulness of the Coombs test, and they call it the "developing test." Diamond and Abelson have recommended a test for the

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detection of the sensitization of babies' blood cells which they call the "reagglutination test."\*

In a preceding paper<sup>4</sup> observations are recorded demonstrating that sera of premature babies as well as many cord sera of full-term babies were characterized by their relative weakness to activate the incomplete Rh antibody, which was in striking contrast to the capacity of adult serum to activate this antibody. With this in mind the following experiments were designed to determine whether normal adult serum would produce agglutination of red blood cells sensitized with the Rh antibody.

#### EXPERIMENTAL

The first four experiments describe typical procedures followed by us for determining sensitization of the blood cells of erythroblastotic babies. A patient, Mrs. Le. (Rh-negative, Group A), with a history of having given birth previously to erythroblastotic children was again delivered of a child exhibiting severe clinical manifestations of this disease. The cord serum of this baby was examined for the presence of free circulating Rh antibodies, the mother's serum taken during delivery being used both for comparison and as a control. The experiment itself was carried out in the following way:

Decreasing amounts of the serum of Mrs. Le. (Columns a and b) as well as cord serum of Baby Le. (Columns c and d) (volume, 0.1 c.c.) were mixed with 0.1 c.c. of a 3 per cent cell suspension of Rh-positive Group O cells. In Columns a and c, physiologic saline solution was used as a diluent for both the serum dilutions and the cell suspensions. In Columns b and d, normal adult serum of Group AB serve as a diluent. The tubes were shaken thoroughly and kept in the water bath at 37° C. for one hour. Then they were centrifuged at medium speed for two minutes. The resulting agglutination is shown in Table I.

TABLE I. AGGLUTINATION OF RH-POSITIVE GROUP O CELLS BY MRS. LE.'S AND BABY LE.'S BLOOD SERA

PATIENTS' SERA	a	b	c	d
	MRS. LE.		CORD SERUM	
	DILUENT			
	SALINE	SERUM	SALINE	SERUM
Undil. 0.1 c.c.	++	+++	-	-
1:2	++	+++	-	-
1:4	++	+++	-	-
1:8	+++	+	-	-
1:16	+++	+	-	-
1:32	+++	+	-	-
1:64	+	+	-	-
1:128	±	±	-	-
1:256	-	-	-	-
0	-	-	-	-

-, No agglutination; ±, faint agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination.

\*According to a personal communication, this test is carried out as follows: "The baby's cells obtained from cord blood are washed twice with saline. A thick drop of the baby's washed cells is placed on a slide and mixed with a slightly larger drop of whole oxalated blood from a known Rh-positive donor of a compatible blood group. The mixture is rocked for two minutes on a viewing box and at the end of two minutes bound or blocked cells will agglutinate the Rh-positive blood of the donor."

The Rh antibody titer of Mrs. Le.'s serum was 128, irrespective of whether saline solution or normal adult serum was used as a diluent. In contrast, no Rh antibodies were demonstrable in the cord serum under the experimental conditions employed. In view of the baby's severe clinical manifestations of erythroblastosis, however, it had to be assumed that the baby's red blood cells were sensitized.

Suspensions of red blood cells in this and in subsequent experiments were prepared from cord blood by the following method:

Umbilical cord blood was allowed to stand until clotted. The clot was then loosened from the sides of the test tube and centrifuged in order to precipitate the clot of cells and separate the serum. After the larger part of the supernatant serum had been decanted, the blood clot was broken up with wooden applicators and the cells resuspended in the small remaining portion of supernatant serum. In other instances, the blood clot was squeezed through several layers of gauze in order to obtain a suitable cell suspension. If the suspensions of blood cells were not homogenous, they were filtered through a paper filter of a type that allowed the passage of red blood cells. (The paper filter was first moistened with cord serum, if available; if not, with saline solution.) Finally, the cell suspension was spun down thoroughly, the supernatant being removed as completely as possible by means of a dropping pipette. The cell sediment was not submitted to any washing procedures.

In order to demonstrate the sensitization of the blood cells under investigation, the following experiment was carried out:

Packed Baby Le.'s cells, 0.0125 c.c., were mixed in test tubes with 0.3 c.c. of: (1) three different normal adult sera (Columns a, b, and c), (2) two normal cord sera (Columns d and e), and (3) cord serum of Baby Le. (Column f). The mixtures were shaken thoroughly and kept for one hour in a water bath at 37° C. They then were shaken slightly and spun down by centrifugation for two minutes at medium speed. The results of this experiment are seen in Table II.

TABLE II. TEST TUBE AGGLUTINATION OF BABY LE.'S CELLS SUSPENDED IN VARIOUS ADULT SERA AND IN CORD SERA, RESPECTIVELY

a	b	c	d	e	f
SERA TESTED					
NORMAL ADULT SERA			NORMAL CORD SERA		CORD SERUM OF BABY LE.
+	+++	±	-	-	-

-, No agglutination. ±, faint agglutination. +, slight agglutination. +++, strong agglutination.

When Baby Le.'s cells were suspended in three normal adult sera in test tubes, varying degrees of agglutination occurred. However, when cord serum, including that of Baby Le., was used as a diluent, there was no agglutination. Obviously, therefore, Baby Le.'s cells were sensitized, but visible agglutination did not occur unless the baby's cells were suspended in adult sera which are suitable for activating the agglutination of Rh-positive cells sensitized by the

incomplete Rh antibody. The specificity of the agglutination observed becomes evident from the following experiment:

Packed cells, 0.01 c.c., obtained from three different sources (Column a, blood cells of Baby Le.; Column b, normal adult blood cells, Group O, Rh-positive; and Column C, normal adult blood cells, Group A, Rh-positive) were mixed, in the first row, with 0.3 c.c. of normal serum Group A and, in the second row, with 0.3 c.c. of Group A citrated plasma originating from the same individual from whom the normal Group A serum was obtained. The experiment was carried out in two parts, Part I being kept in the water bath for one hour at 37° C. and Part II in the icebox for one hour at 4° C. The tubes were then spun down in the centrifuge at medium speed for two minutes. The resulting agglutinations are shown in Table III.

TABLE III. TEST TUBE AGGLUTINATION OF BABY LE.'S CELLS BY NORMAL ADULT SERUM AND PLASMA, RESPECTIVELY

	PART I (1 HR., 37° C.)			PART II (1 HR., 4° C.)		
	a	b	c	d	e	f
	CELLS					
	BABY LE.	BL.	MO.	BABY LE.	BL.	MO.
Serum Group A 0.3 c.c.	+++	-	-	++	-	-
Plasma Group A 0.3 c.c.	++	-	-	±	-	-

- , No agglutination; ±, faint agglutination; ++, marked agglutination; +++, strong agglutination.

The specificity of the agglutination of Baby Le.'s cells suspended either in normal adult serum or in normal adult citrated plasma becomes apparent. Two cell suspensions from normal individuals similarly prepared failed to show any agglutinations. In this experiment agglutination of the baby's cells appears to be stronger at body temperature than at icebox temperature.

The agglutination of Baby Le.'s cells in normal adult serum could also be shown very clearly on the slide. As a matter of fact, the slide technique which was carried out in the following manner proved to be most valuable for demonstrating sensitization of this baby's cells.

With a wax pencil, two rings about 2 cm. in diameter were made on a clean microscope slide. In the center of one ring two large loopfuls (or their equivalent) of packed washed Baby Le.'s cells were placed. In the second ring the same amount of packed normal adult Rh-positive cells of Group O was placed. One large drop of normal adult serum of Group A\* was added to each ring and the mixtures were stirred with wooden applicators. The slide was constantly tilted back and forth by hand, or was placed on a viewing box as recommended by Diamond, and rocked for a few minutes. Frequently five to ten minutes elapsed before agglutination occurred.

An alternative method to the one described consisted of the following: One large drop of adult serum of Group A was placed in each of two rings on a slide. Two wooden applicators were placed in the test tube containing packed

\*Since Baby Le. belonged to Group A, serum of Group A or of Group AB was used in all experiments.

Baby Le.'s cells. The sticks were removed and placed into the first drop of serum on the slide. Sometimes this was repeated, using the other ends of the sticks, in order to insure at least a 10 to 20 per cent cell suspension. Normal Rh-positive Group O cells were added to the second drop of serum in the same manner.

The agglutination of the baby's cells never reached the stage of one solid clump. Instead, agglutination was observed at first only with a magnifying glass, and finally, as time elapsed, ended with the production of many clumps of larger size. Oxalated adult plasma also proved very suitable for demonstrating sensitization. Agglutination of Baby Le.'s cells when taken up in a drop of oxalated plasma on a slide occurred faster and appeared to be even stronger than when taken up in a drop of serum. However, normal cells, too, agglutinated occasionally in oxalated plasma. The addition of one drop of normal saline solution to these latter preparations resulted in a dispersion of the agglutination of the normal cells but did not disturb the agglutination of Baby Le.'s cells. Nevertheless, because of its possible nonspecific effect we feel that oxalated plasma should not be used routinely for this purpose and that serum is the diluent of choice.

Since the cord serum of Baby Le. did not produce agglutination of his own sensitized cells, the following experiment was conducted to determine whether mixtures of Baby Le.'s serum and normal adult serum would induce agglutination of Baby Le.'s cells in view of the fact that this would have important clinical implications. Several mixtures of Baby Le.'s cells with various dilutions of normal adult serum in his own serum were prepared:

- 1 part of normal adult serum of Group A plus 29 parts of cord serum (Baby Le.).
- 1 part of normal adult serum of Group A plus 9 parts of cord serum (Baby Le.).
- 3 parts of normal adult serum of Group A plus 7 parts of cord serum (Baby Le.).
- 1 part of normal adult serum of Group A plus 1 part of cord serum (Baby Le.).
- 3 parts of normal adult serum of Group A plus 1 part of cord serum (Baby Le.).

Two rings about 2 cm. in diameter were drawn on each of four microscope slides with a wax pencil. One large drop of each of the described five mixtures was placed in five separate rings. One drop of undiluted cord serum (Baby Le.) was placed in the sixth ring and one drop of the same normal adult serum undiluted in the seventh ring. The eighth ring contained one drop of 20 per cent albumin solution. To each of these drops, Baby Le.'s packed cells were added by means of wooden applicators as described previously. The mixtures were stirred and constantly rocked on a viewing box and read after ten minutes. Table IV shows the results of this experiment.

TABLE IV. SLIDE AGGLUTINATION OF BABY LE.'S CELLS BY VARIOUS MIXTURES OF CORD SERUM AND NORMAL ADULT SERUM

a	b	c	d	e	f	g	h
SERA TESTED							
SERUM MIXTURES					CORD SERUM	ADULT SERUM	ALBUMIN 20%
I	II	III	IV	V			
-	+	++	+++	++++	-	++++	-

- , No agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination; +++++, very strong agglutination.

Undiluted normal adult serum produced strong agglutination of Baby Le.'s cells, whereas Baby Le.'s own cord serum failed to do so at all. The addition of increasing amounts of adult serum to the cord serum results in mixtures which have an increasing capacity to agglutinate the sensitized cells. It is interesting to note that a mixture consisting of as little as one part of normal adult serum and nine parts of cord serum agglutinated Baby Le.'s cells even though the degree of agglutination was rather weak. Baby Le.'s cells were not agglutinated in 20 per cent albumin solution under these experimental conditions, an observation which we made repeatedly. Obviously, albumin solution was not a suitable medium for bringing out the agglutination of Baby Le.'s sensitized cells in the slide test.

The experimental results obtained with the serum and cells of Baby Le. may be considered to be typical of erythroblastotic babies whose cord sera do not contain any demonstrable free antibodies. The following three experiments deal with a case in which free Rh antibodies were found in the cord serum.

Mrs. Bar. (Group A, Rh negative), whose serum during pregnancy showed a rather high titer of Rh antibodies of the incomplete variety, was delivered at term of a baby exhibiting severe clinical manifestations of erythroblastosis. The baby belonged to Group A and was Rh positive. An exchange transfusion was successfully performed using Rh-negative, Group A blood. A sample of blood was obtained after each infusion of about 60 c.c. of the donor's blood. Nine blood specimens were collected in Wassermann tubes at regular intervals during the exchange transfusion, the first one being taken before the transfusion started, the last one at the end of the transfusion. The first four blood specimens failed to clot, whereas the remaining ones did, possibly due to the heparin used. The baby's serum (or plasma) collected at the beginning of the transfusion was examined for Rh antibodies in the manner described in the first experiment (Table I). An Rh antibody of the incomplete variety was found in a titer of 16. No saline Rh agglutinin was present. (The mother's incomplete Rh antibody titer was quite high at the time of delivery, above 250.)

In the experiments to be described, the nine blood specimens obtained from Baby Bar. during the exchange transfusion were spun down and the supernatant sera were removed from the sediments. Inasmuch as the sediments of Sera 5 through 9 consisted of blood clots, cell suspensions were prepared as described in the case of Baby Le. The cell sediments of Sera 1 through 4 were not treated further and were used as such. None of the cell suspensions was washed.

In order to determine if Baby Bar.'s red blood cells were sensitized by Rh antibodies the following experiment was carried out: With a wax pencil, ten rings were drawn on five slides. In the first nine rings one drop each of the nine serum specimens obtained during the exchange transfusion was placed. One drop of normal adult serum of Group A was put into the tenth ring. Baby Bar.'s cells obtained from the first blood specimen, drawn before the blood transfusion was begun, were then added to each drop of serum by means of wooden applicators. The concentration of blood cells finally obtained



was estimated to be about 20 per cent. The mixtures within each ring were stirred thoroughly and then placed upon the viewing box and constantly rocked. After ten minutes the results were noted and are recorded in Table V.

TABLE V. SLIDE AGGLUTINATION OF BABY BAR.'S SENSITIZED CELLS BY NORMAL ADULT SERUM AND BY VARIOUS SERUM SPECIMENS TAKEN DURING AN EXCHANGE TRANSFUSION

Serum 1	++
Serum 2	-
Serum 3	-
Serum 4	-
Serum 5	-
Serum 6	-
Serum 7	-
Serum 8	-
Serum 9	+
Adult serum	+++

- , No agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination.

The serum of Baby Bar. (Serum 1), when added to its own cells, produced agglutination on the slide. Surprisingly enough, however, Sera 2 to 8, taken during the exchange transfusion, failed to do so. The last serum, 9, again gave a weak agglutination. Normal adult serum of Group A, when mixed with Baby Bar.'s cells, resulted in a strong agglutination. At the present time we are unable to explain why the serum specimens obtained during the exchange transfusion failed to produce any visible agglutination of Baby Bar.'s cells.

It is the obvious goal of an exchange transfusion to replace the baby's blood with normal blood free of Rh antibodies. For that purpose Rh-negative blood is used by most investigators. During the procedure the donor's Rh-negative blood cells are constantly mixed in ever-increasing amounts with the baby's Rh-positive blood cells. The presence or absence of Rh-positive blood cells in the blood specimens taken during exchange transfusions may be considered a rough indicator of the success and extent of the operation. To this end the following experiment was carried out on the series of blood specimens obtained from Baby Bar. during the exchange transfusion.

From the blood sediments of the nine specimens obtained from Baby Bar. during the exchange transfusion 2 per cent blood cell suspensions were prepared in physiologic saline solution. As controls, 2 per cent suspensions of normal Rh-negative and of normal Rh-positive cells were used. In the first row of tubes (Column a) 0.05 c.c. of each of the 2 per cent cell suspensions was mixed with 0.05 c.c. of anti-Rh test serum 1 (anti-D, 85 per cent); in the second row (Column b), with anti-Rh test serum 2 (anti-D, 85 per cent), and in the third row (Column c), with 0.05 c.c. of physiologic saline solution. After being shaken thoroughly, the tubes were kept for one hour at room temperature and then centrifuged at medium speed for two minutes. The resulting macroscopic agglutination is shown in Table VI.

The two Rh test sera secured from different sources strongly agglutinated the Rh-positive cells which were used as controls. The first test serum agglutinated Baby Bar.'s cells taken from the first and second specimens, while cells

TABLE VI. TEST TUBE AGGLUTINATION OF VARIOUS BLOOD CELL SUSPENSIONS TAKEN FROM BABY BAR. DURING EXCHANGE TRANSFUSION BY TWO DIFFERENT ANTI-RH TEST SERA

	a ANTI-RH SERUM 1	b ANTI-RH SERUM 2	c SALINE SOLUTION
Blood cell suspension 1*	++	+++	-
Blood cell suspension 2	+	+++	-
Blood cell suspension 3	-	+++	-
Blood cell suspension 4	-	++	-
Blood cell suspension 5	-	++	-
Blood cell suspension 6	-	+	-
Blood cell suspension 7	-	-	-
Blood cell suspension 8	-	-	-
Blood cell suspension 9	-	-	-
Normal Rh-positive cells	+++	+++	-
Normal Rh-negative cells	-	-	-

-, No agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination.

\*Sample 1 was obtained before any blood was infused; 40 c.c. of blood were withdrawn from the baby and replaced with about 40 c.c. of donor's blood between each subsequent sample.

from the third specimen were negative with this serum. In contrast, the second anti-Rh test serum strongly agglutinated Baby Bar.'s cells up to and including the sixth specimen; blood cells from the seventh specimen failed to show agglutination. Obviously the strength and quality of the Rh test serum used are of great importance in the detection of the baby's Rh-positive cells in blood mixtures resulting from exchange transfusions. The agglutination of the baby's Rh-positive cells by anti-Rh serum seems to be a practical way of testing for their presence in mixtures consisting of both the baby's blood cells and the donor's blood cells as obtained during exchange transfusion. However, the interpretation of this latter observation is not entirely clear. First, the agglutination of the baby's sensitized cells may not have depended solely on the presence of antibodies in the anti-Rh test serum. It has already been pointed out that normal adult serum will induce agglutination of sensitized red blood cells. Second, Baby Bar.'s umbilical cord serum contained a free circulating antibody, suggesting complete sensitization of Baby Bar.'s Rh-positive cells. The question thus arose as to why Baby Bar.'s cells were still subject to agglutination by anti-Rh sera. In order to elucidate these two points, the following experiment was performed.

Twenty circles (about 2 cm. in diameter) were drawn with a wax pencil on several slides. One drop of commercial anti-Rh serum (recommended for the slide technique) was placed in each of ten rings, one drop of normal pooled adult serum of Group A being placed in a second group of ten rings. To the first nine rings of each group packed cells of Baby Bar. prepared from the nine different specimens obtained during the exchange transfusion were added by means of wooden applicators. The tenth ring of each series was used as a control for which normal Rh-positive cells of Group A were used. The slides were placed on the viewing box, rocked, and the resulting agglutination observed after about ten minutes. Table VII shows the findings of this experiment.

The commercial anti-Rh serum agglutinated Baby Bar.'s cells up to the fifth specimen, while cells taken from the sixth specimen failed to show any

TABLE VII. SLIDE AGGLUTINATION OF VARIOUS BLOOD CELL SUSPENSIONS TAKEN FROM BABY BAR, DURING EXCHANGE TRANSFUSION BY ANTI-RH SERUM AND BY NORMAL ADULT SERUM

	a	b
	ANTI-RH SERUM	NORMAL ADULT SERUM
Blood cell suspension 1	++	+++
Blood cell suspension 2	++	+++
Blood cell suspension 3	++	++
Blood cell suspension 4	+	+
Blood cell suspension 5	±	±
Blood cell suspension 6	-	-
Blood cell suspension 7	-	-
Blood cell suspension 8	-	-
Blood cell suspension 9	-	-
Normal Rh-positive cells	++++	-

-, No agglutination; ±, faint agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination; +++++, very strong agglutination.

agglutination. However, normal adult serum also allowed the recognition of the baby's sensitized cells up to the fifth specimen, thus resembling very closely the results obtained with the anti-Rh serum. The fact that the serum containing anti-Rh antibodies was no more effective in agglutinating this particular baby's cells than was the serum without antibodies would lead one to conclude that in addition to Rh antibodies the serum itself is an important factor. Until further evidence is obtained we do not recommend that this test be used as a guide as to the amount of blood that should be given in an exchange transfusion.

#### DISCUSSION

The findings reported in this paper have been essentially confirmed by the last six cases of erythroblastosis which we have studied. All six infants exhibited severe clinical manifestations of the disease. Two died shortly after birth before treatment could be administered, while the other four were treated by means of exchange transfusions and lived.\* In all six cases the mothers were Rh negative and the babies were Rh positive. Further studies are necessary to determine the sensitivity of the described methods and the applicability of this technique to the detection of mild forms of erythroblastosis.

Normal adult serum, active as well as inactive, was found to be the most suitable diluent for bringing about the agglutination of sensitized cells obtained from babies with erythroblastosis fetalis, especially when the slide test was used. Why albumin solutions, 20 per cent as well as 30 per cent, were not suitable for this purpose is not quite clear. However, these latter solutions can be used successfully in the test tube. Oxalated plasma also proved to be a good medium, its only disadvantage being that it frequently produced a non-specific type of pseudoagglutination. Nonspecific reactions with the use of normal adult serum have not been observed while examining the cases under consideration. However, in the study of a larger series of normal cord bloods, pseudoagglutination (rouleaux formation) was occasionally encountered, thus making it necessary to observe the slides microscopically. Therefore, from a diagnostic standpoint, the agglutination obtained must be interpreted with great

\*We are very much indebted to Dr. Douglas P. Arnold who performed the four exchange transfusions and furnished us with the material for these studies.

care. The presence of even small amounts of saline solution prevented or reduced the agglutination of the baby's sensitized cells. For this reason, packed unwashed cells were used with undiluted adult serum. Wiener<sup>7</sup> reported the agglutination of red blood cells of erythroblastotic babies when these cells were suspended in their own cord sera. In our experience, cord sera are considerably inferior to normal adult sera in this capacity. One case exhibiting a somewhat different feature from the cases described herein seems to be worthy of mention in this connection. In this instance the preparation of a cell suspension from the baby's cord blood clot was more difficult than usual because of a marked degree of hemolysis. The cells were found to be Rh positive and were considered to belong to Group A<sub>2</sub>B. The B property of the baby's cells was strongly and definitely agglutinated by an anti-B isoagglutinin; the presence of the A property, however, was somewhat questionable in spite of the fact that both test sera, produced by immunizing donors with blood group-specific substances, were very potent.<sup>8</sup> When normal serum of Group AB was added to the baby's blood cells, the same weak type of agglutination occurred as was obtained with test serum of Group B, indicating that the agglutination of these cells was not due to the interaction between an A antibody and an A factor, but to the fact that the baby's cells were sensitized with an Rh antibody, thus exhibiting agglutination when suspended in normal adult serum. This child's mother belonged to Group O, and one could have construed very easily an exemption to the Bernstein law of inheritance of the blood groups.\* The baby's cells, even when suspended in saline solution and examined under the microscope, already showed in each field occasional clumps consisting of five to ten cells. When the baby's packed cells were added to normal cord serum on a slide, macroscopically visible agglutination occurred. The baby's own cord serum was not available.

As a result of the difficulty experienced in the determination of this baby's blood group, one wonders if similar difficulties might be encountered in the determination of the Rh type of erythroblastotic babies. In many instances the baby's Rh type is considered to be positive because agglutination of the cells occurs following the addition of an anti-Rh serum. The question arises as to whether agglutination of red blood cells from erythroblastotic babies by an anti-Rh serum is actually caused by the Rh antibody proper, or whether it is merely due to the addition of normal adult serum. Surprisingly enough, *agglutination takes place even in instances in which the baby's cord serum contains free circulating Rh antibodies which, one might assume, had combined with all the available Rh antigens present in the baby's cells.* The interpretation of agglutination tests with the blood cells of erythroblastotic babies, therefore, needs careful observation from the standpoint of specificity.

Adult serum of any group can be used to bring about agglutination of the baby's sensitized cells by the slide technique, provided that the blood group

\*In order to avoid this statement being misused legally to question the validity of Bernstein's rule, it should be added that this baby, if it had survived, would soon have replaced its sensitized cells with normal cells and would have shown its true blood group a few weeks after delivery.

corresponds to that of the cells. Since there are considerable individual variations in the suitability of individual adult sera, pools can be prepared from adult sera of the same blood group. If the baby belongs to Group O, no attention need be paid to the blood group of the adult serum to be used. In cases where the baby's blood group is unknown or is questionable, serum of Group AB is the safest one to employ.

From the standpoint of treatment, the observations reported in Table IV may be significant. This experiment showed that the addition of as little as 1 part of adult serum to 9 parts of the baby's cord serum induced macroscopically visible agglutination of the baby's cells on the slide. Thus the transfusion of 30 to 50 c.c. of adult whole blood into a newborn baby might be sufficient to enhance the *in vivo* agglutination of the baby's sensitized cells, resulting in further damage to them. Experimental evidence supporting this theoretical possibility, however, has not yet been found. What happens to the activating properties of adult plasma following transfusion into an erythroblastotic baby still remains unknown.

#### CONCLUSION

A simple technique for the demonstration of sensitization of the red blood cells in an erythroblastotic baby is described. Agglutination occurs when the packed blood cells of an erythroblastotic baby are suspended in normal adult serum. This test can be carried out either in test tubes or on microscopic slides, the use of the latter proving more sensitive in our hands. The results obtained from this test indicate that the baby's cells are sensitized by the incomplete type of Rh antibody which does not cause agglutination in saline solution, and only slight agglutination if any, in cord serum. Blood specimens taken from erythroblastotic babies during exchange transfusions were examined for the continued presence of sensitized cells. Some practical aspects concerning the validity and specificity of Rh type and blood group determinations of erythroblastotic babies, as well as the treatment of these babies by blood transfusion, are discussed.

#### REFERENCES

1. Carter, Bettina B., and Loughrey, J.: A Method of Demonstrating Anti-Rh Agglutinins in Cases of Erythroblastosis Fetalis, *Am. J. Clin. Path.* 15: 575-576, 1945.
2. Coombs, R. R. A., Mourant, A. E., and Race, R. R.: A New Test for the Detection of Weak and Incomplete Rh Agglutinins, *Brit. J. Exper. Path.* 26: 255-266, 1945.
3. Coombs, R. R. A., Mourant, A. E., and Race, R. R.: In-Vivo Isosensitization of Red Cells in Babies With Hemolytic Disease, *Lancet* 1: 264-270, 1946.
4. Coombs, R. R. A., and Race, R. R.: Further Observations on "Incomplete" or "Blocking" Rh Antibody, *Nature*, London 156: 233-234, 1945.
5. Hill, J. M., and Haberman, Sol: Demonstration of Rh Antibodies in the Newborn and Further Evidence of the Pathogenesis of Erythroblastosis, *J. LAB. & CLIN. MED.* 31: 1053-1066, 1946.
6. Witebsky, Ernest, Rubin, Mitchell I., and Blum, Livia: Studies in Erythroblastosis Fetalis. I. Activation of the Incomplete Rh Antibody by the Blood Serum of Full-Term and Premature Newborn Infants, *J. LAB. & CLIN. MED.* 32: 1330, 1947.
7. Wiener, A. S.: Conglutination Test for Rh Sensitization, *J. LAB. & CLIN. MED.* 30: 662, 1945.
8. Witebsky, E., Klendshoj, N. C., and McNeil, C.: Potent Typing Sera Produced by Treatment of Donors With Isolated Blood Group Specific Substances, *Proc. Soc. Exper. Biol. & Med.* 55: 167-170, 1944.

## THE URINARY EXCRETION OF PTEROYLGLUTAMIC ACID AND CERTAIN RELATED COMPOUNDS

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IT BECAME evident in the early stages of the investigations of *Lactobacillus casei* factor (folie acid) that only small amounts were excreted in the urine of normal human subjects.<sup>1, 2</sup> The amount excreted daily was estimated to be less than 1 per cent of the probable dietary intake.<sup>2</sup>

These observations indicated that dietary pteroylglutamic acid was probably broken down before excretion. Later, pure pteroylglutamic acid (PGA) became available for administration as a supplement. It then appeared that the amount of pteroylglutamic acid excreted in the urine increased sharply as the dosage was raised, indicating that the capacity of the body to break down pteroylglutamic acid was limited. A corollary of these observations was that pterioic acid did not appear to be excreted as a breakdown product of dietary pteroylglutamic acid because, on a normal unsupplemented diet, the potency of the urine was low when measured either by *Streptococcus faecalis* R<sup>1</sup> or *L. casei*.<sup>2</sup>

In a study carried out with young men on a normal diet, it was found that the average daily urinary folie acid excretion was about 4  $\mu$ g as measured by assay with *Str. faecalis* R.<sup>3</sup> Supplementation with a very small amount (90 $\mu$ g) of pteroylglutamic acid daily did not increase the urinary excretion.<sup>4</sup>

The daily urinary excretion of pteroylglutamic acid was found to be 2 to 4  $\mu$ g, rarely exceeding 5  $\mu$ g, on ordinary diets.<sup>5</sup> Oral or parenteral administration of pteroylglutamic acid led within twenty-four hours to an excretion of 15 to 75 per cent of the amount administered, depending on the size of the dose; one individual excreted 16 per cent of an intramuscular dose of 1 mg., while with a dosage of about 10 mg. daily, either orally or parenterally, the excretion usually ranged between 35 and 50 per cent. When pteroyltriglutamic acid was administered to a patient with sprue, assay of the urine with *L. casei* and *Str. faecalis* R indicated that pteroylglutamic acid was liberated from pteroyltriglutamic acid and excreted in considerable quantities.

Doses of 5 to 16 mg. of pteroylglutamic acid were fed to nine normal subjects and nine hospital patients.<sup>6</sup> The normal subjects before receiving pteroylglutamic acid had an average daily urinary excretion of between 2 and 3  $\mu$ g of pteroylglutamic acid. They excreted an average of 28.5 per cent of the administered dose. Most of the excretion took place between the second and eighth hour after dosage. The hospital patients excreted much lower percentages of the administered dose.

Studies with six normal subjects<sup>7</sup> showed that only about 3  $\mu$ g of pteroylglutamic acid, as measured by assay with *L. casei*, were present in the twenty-

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four hour urine prior to administration of pteroylglutamic acid. When 4 mg. of the compound were given, about 32 per cent of the dose was excreted in the urine in twenty-four hours.

## EXPERIMENTAL

Normal adult men were used as subjects. Since it was found that on ordinary diets the pteroylglutamic acid (PGA) content of the urine was very low (between 5 and 10 millimicrograms per milliliter) no attempt was made to control the diet and, except when very small test doses were used, it was unnecessary to apply a correction for the basal value of the urine.

Samples were collected in brown bottles and preserved under toluene in the refrigerator. Folic acid was assayed against a pteroylglutamic acid standard with *Str. faecalis* R,<sup>s</sup> using purine, pyrimidine, amino acid, and vitamin supplements as described by Teply and Elvehjem.<sup>9</sup>

In the first series, the urinary excretion of pteroylglutamic acid was studied following the administration of various dosage levels of a solution of the sodium salt. Two experiments were included in which the excretion of the free acid suspended in water or oil was measured. The results, shown in Table I,

TABLE I. EFFECT OF DOSAGE AND MODE OF ADMINISTRATION ON URINARY EXCRETION OF PTEROYLGLUTAMIC ACID

FORM ADMINISTERED	AMOUNT PGA TAKEN (MG.)	COLLECTION PERIOD OF URINE (HR.)	ROUTE OF ADMINISTRATION	PER CENT OF DOSE EXCRETED IN URINE	AVERAGE
Solution of Na salt	0.1	6	Oral	0, 0, 0.9, 0.6	0.4
Solution of Na salt	0.5	6	Oral	0, 12, 25, 19, 3, 14	12
Solution of Na salt	1.0	6	Oral	10, 25, 26, 26	22
Solution of Na salt	2.0	6	Oral	32, 36, 38, 51, 56	43
Solution of Na salt	5.0	6	Oral	45, 49, 49, 44, 48, 52	49
Solution of Na salt	15.0	24	Oral	48, 46, 57, 56	77
Solution of Na salt	5.0	6	Intravenous	81, 92, 57	60
Suspension in water	5.0	6	Oral	51, 54, 72, 64	34
Suspension in oil*	5.0	6	Oral	35, 31, 39, 32, 35	20
				15, 15, 17, 24, 28	

\*Mazola oil.

indicated that the proportion which was excreted increased as the dose was raised. The greater part of the excretion took place in the first six hours. A small part of the administered dose was excreted in the next eighteen hours. This is illustrated in the data in Table II. An average of 53 per cent of a

TABLE II. TIME RELATIONS IN THE URINARY EXCRETION OF FOLIC ACID FOLLOWING THE ORAL ADMINISTRATION OF 5 MG. OF SODIUM PTEROYLGLUTAMATE

SUBJECT	PER CENT OF DOSE EXCRETED		(% OF 24-HOUR EXCRETION) EXCRETION IN FIRST 6 HOURS
	0 - 6 HR.	6 - 24 HR.	
1	48	2	96
2	48	5	91
3	52	6	90
4	41	4	91
2	49	9	84
5	47	8	85

dose of 5 mg. was excreted in twenty-four hours, and an average of 90 per cent of this excretion took place in the first six hours. After twenty-four hours the urinary folic acid content returned to the basal level.

In the next series, the excretion of folic acid in the urine was studied following the administration of pteroyltriglutamic acid (pteroyl- $\gamma$ -glutamyl- $\gamma$ -glutamyl glutamic acid).<sup>10</sup> This compound has about 3 per cent of the activity of pteroylglutamic acid in the *Str. faecalis* R assay. Urinary excretion values in excess of the total microbiologic activity of the administered dose were presumed to indicate that pteroyltriglutamic acid had been excreted as pteroylglutamic acid. The results, which are given in Table III, led to the conclusion that pteroyltriglutamic acid was readily hydrolyzed in the body to pteroylglutamic acid because the urinary excretion values following the administration of pteroyltriglutamic acid were very similar to those observed with pteroylglutamic acid.

TABLE III. AMOUNTS OF FOLIC ACID ACTIVITY FOUND PRESENT IN THE URINE FOLLOWING ADMINISTRATION OF 7.9 MG. OF PTEROYLTRIGLUTAMIC ACID (EQUIVALENT TO 5 MG. OF PTEROYLGLUTAMIC ACID) WHEN ADMINISTERED AS SOLUTION OF SODIUM SALT

SUBJECT	ROUTE OF ADMINISTRATION	PER CENT OF DOSE EXCRETED (MEASURED AS PGA)		EXCRETION IN FIRST 6 HOURS (% OF 24-HOUR EXCRETION)
		0 - 6 HR.	6 - 24 HR.	
4	Oral	37	9	80
5	Oral	51	11	83
6	Oral	38	5	91
7	Oral	62	12	84
4	Intravenous	58	11	84
4	Intravenous	51	10	84
2	Intravenous	45	7	85

There was some indication of a lag in excretion as evidenced by the proportion of the biologic activity excreted in the 6- to 24-hour period being greater with pteroyltriglutamic acid than with pteroylglutamic acid. An average of 15 per cent of the twenty-four hour excretion was found in the urine collected from the sixth to the twenty-fourth hour following dosage with pteroyltriglutamic acid as compared with 10 per cent following dosage with pteroylglutamic acid. The excretion rate, measured as pteroylglutamic acid, appeared to be the same following dosage by either the oral or intravenous route, which could lead to the assumption that the breakdown of pteroyltriglutamic acid to pteroylglutamic acid in the body could take place with equal rapidity whether or not the triglutamic compound passed into the body through the gastrointestinal tract.

Experiments were also made with pteroyldiglutamic acid (pteroyl- $\alpha$ -glutamyl glutamic acid).<sup>10</sup> This compound has practically no activity in stimulating the growth of either *L. casei* or *Str. faecalis* R, so that any substantial increase of microbiologic activity in the urine could be attributed to pteroylglutamic acid formed by breakdown of pteroyldiglutamic acid in the body. The results are in Table IV. The data indicate that the body possesses the ability to liberate pteroylglutamic acid from pteroyldiglutamic acid. Even more than in the case of pteroyltriglutamic acid, there was a lag in the rate of excretion so that a comparatively large but variable proportion of the administered dose, measured



TABLE IV. EXCRETION OF FOLIC ACID IN THE URINE FOLLOWING THE ADMINISTRATION OF 6.5 MG. OF PTEROYLDIGLUTAMIC ACID (STOICHIOMETRICALLY EQUIVALENT TO 5 MG. OF PGA) AS SODIUM SALT

SUBJECT	ROUTE OF ADMINISTRATION	PER CENT OF DOSE EXCRETED (MEASURED AS PGA)		EXCRETION IN FIRST 6 HOURS (% OF 24-HOUR EXCRETION)
		0 - 6 HR.	6 - 24 HR.	
2	Oral	21	7	75
3	Oral	37	6	88
5	Oral	33	5	87
8	Oral	42	6	88
2	Oral	11		
3	Oral	42		
5	Oral	30		
8	Oral	43		
2	Intravenous	15	9	62
4	Intravenous	14	15	48
5	Intravenous	14	11	56

as pteroylglutamic acid, was found in the urine during the period between six and twenty-four hours after dosage. The effect was especially marked following intravenous administration.

The last compound studied was the diamide of pteroylglutamic acid.<sup>11</sup> This substance does not appreciably stimulate the growth of *L. casei* or *Str. faecalis* R. No increase in the urinary pteroylglutamic acid level was found in twenty-four hour samples following the oral administration of 10 mg. doses to three subjects. The compound was prepared from *p*-aminobenzoylglutamic acid diamide, 2,3-dibromopropionaldehyde, and 2,4,5-triamino-6-hydroxypyrimidine. Hydrolysis of the compound by autoclaving with 0.4 N NaOH for one hour at 130° C. resulted in the liberation of 69 per cent of the calculated quantity of pteroylglutamic acid, as measured by assay with *Str. faecalis* R.

#### DISCUSSION

The apparent ability of the body to hydrolyze pteroyldiglutamic acid, as evidenced by the urinary excretion data in the present investigation, is in contrast to the microbiologic inactivity of this substance but may be in harmony with its reported effect in producing a remission in macrocytic anemia.<sup>12</sup> The inactivity of pteroyldiglutamic acid for *L. casei* and *Str. faecalis* R may indicate that these organisms do not possess a "conjugase" system which is effective in hydrolyzing the peptide linkage in pteroyldiglutamic acid between the terminal glutamic acid and the  $\alpha$ -carboxyl group of the glutamic acid radicle of the pteroylglutamic acid moiety of the molecule. In contrast, pteroyltriglutamic acid, in which the glutamyl radicles are linked through their  $\gamma$ -carboxyl groups, is fully active for *L. casei* which may mean that this organism has a "conjugase" system which hydrolyzes the  $\gamma$  linkage between glutamyl groups. An alternative explanation would be that *L. casei* utilizes both pteroylglutamic acid and pteroyltriglutamic acid, but not pteroyldiglutamic acid, as precursors of more complex substances.

The nature of the possible breakdown products of pteroylglutamic acid that are excreted in the urine is a matter of speculation. The presence of

xanthopterin in normal urine has been repeatedly noted and it is logical to infer that this substance may be formed by the breakdown of pteroylglutamic acid.\*

The present study indicated that pteroyltriglutamic acid is rapidly absorbed following administration, for pteroylglutamic acid was excreted in the urine at approximately the same rate following either oral or intravenous administration of pteroyltriglutamic acid. The data also indicate that, as previously noted,<sup>5</sup> pteroyltriglutamic acid is readily hydrolyzed to pteroylglutamic acid in the body. The site of this breakdown is not known, but preparations made from various organs have been shown to have "vitamin B<sub>12</sub> conjugase" activity<sup>13</sup> as indicated by liberation of pteroylglutamic acid from pteroylheptaglutamic acid. Certain enzyme preparations of this type, prepared from chicken pancreas, have been found readily to liberate pteroylglutamic acid from pteroyltriglutamic acid.<sup>14</sup>

The limited data in Table I appeared to indicate that, with a 5 mg. dose of pteroylglutamic acid, an oily vehicle appeared to depress the excretion and hence possibly the uptake of an oral dose; also, the rate of excretion following the administration of a solution of the sodium salt was somewhat higher than when a suspension of the free acid was used.

#### CONCLUSIONS

1. The urinary excretion of folic acid, as measured by assay with *Str. faecalis* R, was studied with normal subjects receiving various dosages of pteroylglutamic acid (PGA), pteroyltriglutamic acid, and pteroyldiglutamic acid.

2. The proportion of the administered dose of pteroylglutamic acid which was excreted was found to increase as the dose was raised.

3. With pteroylglutamic acid most of the excretion took place within the first six hours, a small part of the administered dose was excreted in the next eighteen hours, and after twenty-four hours the excretion returned to the basal level of between 5 and 10 millimicrograms per milliliter.

4. The microbiologic assay data indicated that pteroyltriglutamic acid was readily hydrolyzed to pteroylglutamic acid in the body when administered by either the oral or intravenous route.

5. Similar results were observed following the administration of pteroyldiglutamic acid. A slight lag in the excretion rate observed with pteroyltriglutamic acid was somewhat more marked in the case of pteroyldiglutamic acid.

6. No increase in the urinary pteroylglutamic acid level was found following the administration of the diamide of pteroylglutamic acid.

The collaboration of Mr. J. Van Meter in assisting with the microbiologic assays is gratefully acknowledged.

\*Recently it has been reported by Denko (paper presented at the One Hundred Twelfth Meeting of the American Chemical Society, New York, September, 1917) that, following oral administration of 10 mg., the apparent urinary excretion of pteroylglutamic acid as measured with *L. casei* in some cases far exceeded the dose. No such observation was made in the present study, using *Str. faecalis* R.

## REFERENCES

1. Wright, L. D., McMahon, R., Jr., Cheldelin, V. H., Taylor, A., Snell, E. E., and Williams, R. J.: The "B Vitamins" in Normal Tissues (Autolysates), University of Texas Publication No. 4137, p. 38, 1941.
2. Wright, L. D., and Welch, A. D.: The Production of Folic Acid by Rat Liver in Vitro, *Science* 88: 179, 1943.
3. Denko, C. W., Grundy, W. E., Porter, J. W., Berryman, G. H., Friedemann, T. E., and Youmans, J. B.: The Excretion of B-Complex Vitamins in the Urine and Feces of Seven Normal Adults, *Arch. Biochem.* 10: 33, 1946.
4. Denko, C. W., Grundy, W. E., Wheeler, N. C., Henderson, C. R., Berryman, G. H., Friedemann, T. E., and Youmans, J. B.: The Excretion of B-Complex Vitamins by Normal Adults on a Restricted Intake, *Arch. Biochem.* 11: 109, 1946.
5. Suarez, R. M., Welch, A. D., Heinle, R. W., Suarez, R. M., Jr., and Nelson, E. M.: Effectiveness of Conjugated Forms of Folic Acid in the Treatment of Tropical Sprue, *J. LAB. & CLIN. MED.* 31: 1294, 1946.
6. Steinkamp, R., Shukers, C. F., Totter, J. R., and Day, P. L.: Urinary Excretion of Orally Administered Pteroylglutamic Acid, *Proc. Soc. Exper. Biol. & Med.* 63: 556, 1946.
7. Swendseid, M. E., Bird, O. D., Brown, R. A., and Bethell, F. H.: Metabolic Function of Pteroylglutamic Acid and Its Hexaglutamyl Conjugate, *J. LAB. & CLIN. MED.* 32: 23, 1947.
8. Landy, M., and Dicken, D. M.: A Microbiological Assay Method for Six B Vitamins Using *Lactobacillus Casei* and a Medium of Essentially Known Composition, *J. LAB. & CLIN. MED.* 27: 1086, 1942.
9. Teply, L. J., and Elvehjem, C. A.: The Titrimetric Determination of "Lactobacillus Casei Factor" and "Folic Acid," *J. Biol. Chem.* 157: 303, 1945.
10. Boothe, J. H., Semb, J., Mowat, J. H., Waller, C. W., Hutchings, B. L., Angier, R. B., Stokstad, E. L. R., and Subbarow, Y.: In press.
11. Upham, S., and others: Unpublished observations.
12. Spies, T. D., Stone, R. E., and Toca, R. L.: The Anti-Anemic Properties of Pteroylglutamylglutamic Acid, *South. M. J.* 40: 175, 1947.
13. Bird, O. D., Bressler B., Brown, R. A., Campbell, C. J., and Emmett, A. D.: The Microbiological Assay of Vitamin B<sub>6</sub> Conjugate, *J. Biol. Chem.* 159: 631, 1945.
14. Stokstad, E. L. R., and others: Unpublished observations.

# ANOMALOUS FINDINGS IN PENICILLIN LEVEL DETERMINATIONS IN URINE

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## INTRODUCTION

NUMEROUS investigators (including Florey and co-workers,<sup>2</sup> Rammelkamp and associates, 1943,<sup>3, 4</sup> Free and co-workers, 1944,<sup>5</sup> Mutch and Rewell, 1945,<sup>6</sup> McDermott and associates, 1946,<sup>7</sup> and Eagle, 1947<sup>8</sup>) have shown that from 60 to 100 per cent of the penicillin administered in a single dose may be voided during the first four hours after injection.

However, in the course of determining penicillin levels in a series of urine specimens obtained from patients under treatment for syphilis, unexpectedly high values were observed in a number of urines collected up to the fourth hour following the administration of penicillin. This report deals with the results obtained from a study of these specimens tested for streptococidal action at regular intervals over a three-month period. Since these findings were in excess of what could be explained by the dose injected, some of the factors influencing the test also will be discussed.

## MATERIALS

The urines tested were sent to the laboratory for routine penicillin assay.\* All specimens were refrigerated from time of collection, although a delay of several days until time of assay was frequent. The volume of the samples varied from 50 to 100 milliliters. Unfortunately, the exact volume voided at one time was recorded in only a few instances.

## METHODS

Assays were made by the Rammelkamp method.<sup>9</sup> The samples were filtered through a Seitz filter immediately upon reception at the laboratory, to insure sterility. Before assay, appropriate preliminary dilutions of the urine were made in phosphate buffer pH 6.0, so that an end point would be obtained by the use of not more than fifteen serial dilutions.

The test organism was a Group A hemolytic streptococcus, Strain 98.† This strain was regularly sensitive to between 0.0039 and 0.0078 Oxford units of penicillin per milliliter.

The sample and culture dilutions were made in beef heart broth pH 7.0 to which 50 mg. per liter of para-aminobenzoic acid had been added. The tests and dilution tubes were kept in an ice bath during the execution of the test.

## EXPERIMENTAL RESULTS

None of the specimens of urine tested before treatment was initiated showed streptococidal action equivalent to more than 0.04 unit of penicillin.

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\*By Dr. Alfred Cohn, Venereal Disease Research Division.

†Obtained through the courtesy of Dr. Rammelkamp.

Furthermore, 249 specimens collected from 5 to 143 hours following drug administration showed both reproducible and reasonable assay values. However, these conditions did not prevail in many specimens collected up to the fourth hour following treatment. Anomalous findings were first observed in this group of specimens in a series of six individuals tested in May, 1946. In the initial assay seven of twelve specimens showed levels from five to one thousand times higher than was anticipated. During a period of five weeks' storage another seven of twelve specimens showed an increase in titer from ten to one hundred fold over the initial test. Only one specimen failed to show increased bactericidal action at some time during this period. These results were observed when the subjects were given penicillin in saline, as well as in water and oil emulsion.

Tables I and II show typical protocols. Patient D. B. (Table I) received one injection of 150,000 Oxford units of penicillin in saline solution. Specimens were collected two, four, eight, twelve, and twenty-four hours following the administration of the antibiotic. These samples were tested at weekly intervals over a period of one hundred days. In instances where more than one test was made during a given period of time, the figures represent an average of the results. All figures represent units per milliliter. Each individual specimen contained between 50 and 100 milliliters. The two-hour sample showed a recovery of 4,000 units of penicillin per milliliter. This would mean that the entire sample contained between 200,000 and 400,000 Oxford units, which was more than the amount administered. Furthermore, during the third week of storage, assays showed that the level per milliliter was equivalent to the entire dose injected, and the level increased tenfold more during the following three weeks. Subsequently it dropped to anticipated levels. Specimens collected on the fourth, eighth, twelfth, and twenty-fourth hour showed regular and reproducible results.

TABLE I. PENICILLIN LEVEL DETERMINATION (PATIENT D. B.; DIAGNOSIS, SYPHILIS; PENICILLIN ADMINISTERED, 150,000 O. U. IN SALINE)

DAYS' STORAGE (5° C.)	TIME OF URINE COLLECTION AFTER ADMINISTRATION OF PENICILLIN (HR.)				
	2 (UNITS RE- COVERED/ML.)	4 (UNITS RE- COVERED/ML.)	8 (UNITS RE- COVERED/ML.)	12 (UNITS RE- COVERED/ML.)	24 (UNITS RE- COVERED/ML.)
0	--	--	--	--	--
1 to 7	4,000	62	2.0	0.80	0.04
8 to 14	--	--	--	--	--
15 to 21	160,000	250	5.0	0.62	0.04
22 to 40	1,000,000	80	2.5	0.62	0.04
59 to 78	1,300	65	5.0	--	--
100	500	40	2.5	0.31	--

Patient D. (Table II) received one dose of 1,000,000 Oxford units of penicillin in saline solution. Again more antibiotic action than would be expected from the treatment given was found in the two- and four-hour samples. Similar observations were made on tests made during the first five weeks after collection. However, these same specimens showed normal levels from the sixth through the fourteenth week. Tests on the eight-, twelve-, and twenty-four hour specimens again showed regular and reproducible results.

TABLE II. PENICILLIN LEVEL DETERMINATION (PATIENT D.; DIAGNOSIS, SYPHILIS; PENICILLIN ADMINISTERED, 1,000,000 O. U. IN SALINE)

DAYS' STORAGE (5° C.)	TIME OF URINE COLLECTION AFTER ADMINISTRATION OF PENICILLIN (HR.)				
	2 (UNITS RE- COVERED/ML.)	4 (UNITS RE- COVERED/ML.)	8 (UNITS RE- COVERED/ML.)	12 (UNITS RE- COVERED/ML.)	24 (UNITS RE- COVERED/ML.)
0	--	--	--	--	--
1 to 7	2,000,000+	8,000	125	4.0	4.00
8 to 14	--	--	--	--	--
15 to 21	400,000	400,000+	125	5.0	1.25
22 to 35	2,000,000+	25,000	--	--	--
36 to 60	6,200	800	25	2.5	0.62
61 to 100	5,000	75	40	2.5	--

In October and November, 1946, the one-hour urine specimens of another six subjects who received a single dose of 1,000,000 Oxford units of penicillin in water and oil emulsion were studied. As before, the total volume varied between 50 and 100 ml. per sample. Table III shows the results obtained per milliliter in four typical instances. The general pattern of the early specimens tested in May was repeated. Specimens obtained from these individuals immediately preceding treatment showed no bactericidal activity. Furthermore, the activity of the one-hour specimens was completely destroyed by the use of the penicillinase broth.

TABLE III. UNITS OF PENICILLIN RECOVERED IN URINE COLLECTED FROM SYPHILITIC SUBJECTS ONE HOUR AFTER THE ADMINISTRATION OF 1,000,000 O. U. IN WATER AND OIL EMULSION\*

STORAGE (DAYS)	PATIENT			
	1 (UNITS/ML.)	2 (UNITS/ML.)	3 (UNITS/ML.)	4 (UNITS/ML.)
4 to 7	25,000	6,200	340,000	80,000
8 to 14	2,500,000	620,000	--	--
15 to 21	--	--	--	--
22 to 35	12,500	12,500	800	1,600
36 to 100	8,000	2,000	600	700

\*Squibb penicillin 12003-1; expiration date, April 1, 1947.

The possibility that certain specimens might contain a substance capable of producing a synergistic effect in the presence of penicillin was then investigated. Accordingly, 10,000 Oxford units of a commercial penicillin per milliliter were added to a 10 c.c. portion of pretreatment urine. This sample was then tested at the same time as a specimen collected from this same subject one hour after treatment was started.

Results of these comparative tests are shown in Table IV. The one-hour specimens again showed potentiated bactericidal activity, while the pretreatment urine to which 10,000 Oxford units of penicillin per milliliter were added showed regular and reproducible results which were similar to the result obtained when an equivalent amount of penicillin was diluted with phosphate buffer at pH 6.0. Again the untreated pretreatment urine showed no bactericidal activity, and the addition of penicillinase to either the one-hour urine sample or the penicillin in buffer solution destroyed the activity of the test sample.

TABLE IV. UNITS OF PENICILLIN RECOVERED FROM PRETREATMENT URINE TO WHICH PENICILLIN WAS ADDED AFTER EXCRETION COMPARED WITH LEVELS FOUND IN URINE OF THE SAME PATIENT COLLECTED ONE HOUR AFTER ADMINISTRATION OF 1,000,000 O. U. IN WATER AND OIL EMULSION\*

Test Fluid	TIME OF STORAGE AT 5° C.				
	0 DAYS (UNITS RECOV- ERED/ML.)	1 TO 7 DAYS (UNITS RECOV- ERED/ML.)	8 TO 14 DAYS (UNITS RECOV- ERED/ML.)	15 TO 21 DAYS (UNITS RECOV- ERED/ML.)	22 TO 35 DAYS (UNITS RECOV- ERED/ML.)
Urine collected one hour following the administration of 1,000,000 O. U. in water and oil emulsion	--	25,000	2,500,000	12,500	8,000
Pretreatment urine + 10,000 O. U./ml.	10,000	10,000	10,000	10,000	10,000
Controls					
Buffer pH 6.0 + 10,000 O. U. penicillin/ml.	10,000	10,000	10,000	10,000	10,000
Untreated pretreatment urine	0	0	0	0	0
One-hour urine + penicillinase	0	0	0	0	0
Penicillin in buffer pH 6.0 + penicillinase	0	0	0	0	0

\*Squibb penicillin 12005-1; expiration date, April 1, 1947.

It may be considered that these results were caused by a technical error in the method of dilution of the test samples. Experiments were therefore set up using different initial dilutions, different dilution volumes, and different diluents. No significant variations were observed.

To ascertain whether the high levels were caused by experimental accidents, twenty-four different one-hour specimens\* were divided each in three aliquot parts and tested at regular intervals for a period of six weeks. Again, there was never more than a twofold variation between the different portions of any one specimen.

It was thought that the delay of twenty-four or more hours between the time of collection of the specimens and the time of assay might be a contributing factor in producing the phenomenon of enhanced bactericidal activity. To investigate this possibility, the previously mentioned twenty-four specimens collected one hour after the administration of 1,000,000 O.U. of penicillin in Pendil-beeswax were filtered and tested within one hour after excretion. All tests were carried out in triplicate over a six-week period. During this period there were no significant changes in titer in any of the twenty-four samples.

## COMMENTS AND CONCLUSIONS

In a study of sixty-seven early specimens of urine anomalous findings have been observed in thirty instances. These results did not appear to be caused by the usual technical errors in experimental accidents. Furthermore, the

\*Twenty-one specimens from patients receiving 1,000,000 O.U. Heyden penicillin #1276 (expiration date, Aug. 1, 1948) diluted in Pendil-beeswax; three specimens from patients receiving 1,000,000 O.U. Parke Davis penicillin #02314A (expiration date, July, 1948) diluted in Pendil-beeswax.

phenomenon of increased bactericidal action was not observed in any of the 249 specimens collected more than four hours after treatment.

No explanation for this phenomenon has been found. It is reported, at this time, to call attention to irregularities that may be encountered in attempting to make a quantitative estimate of the penicillin content of early urines.

We wish to thank Dr. Alfred Cohn and Dr. Isaak Grunstein, of the Venereal Disease Research Clinic, for their cooperation in obtaining early specimens of urine, and Mrs. Shirley Tove and Miss Mildred Schleimer for technical assistance.

#### REFERENCES

1. Abraham, E. P., Chain, E., Fletcher, C. M., Gardiner, H. D., Heatley, N. G., Jennings, M. A., and Florey, H. W.: *Further Observations on Penicillin*, *Lancet* 2: 177-189, 1941.
2. Rammelkamp, C. H., and Keefer, C. S.: *The Absorption and Distribution of Penicillin*, *J. Clin. Investigation* 22: 425, 1943.
3. Rammelkamp, C. H., and Bradley, S. E.: *Excretion of Penicillin in Man*, *Proc. Soc. Exper. Biol. & Med.* 53: 30-32, 1943.
4. Free, A. H., Leonards, J. R., McCullough, D. R., and Biro, B. E.: *The Urinary Excretion of Penicillin After Oral Administration in Normal Human Subjects*, *Science* 100: 431, 1944.
5. Mutch, N., and Rewell, R. E.: *Penicillin by Inhalation*, *Lancet* 21: 1650, 1945.
6. McDermott, W., Bunn, P. A., Benoit, M., Dubois, R., and Reynolds, M. E.: *The Absorption, Excretion, and Destruction of Orally Administered Penicillin*, *J. Clin. Investigation* 25: 190-210, 1946.
7. Eagle, Harry: *The Varying Blood Levels Afforded by Penicillin, F, G, K, and X in Rabbits and Man*, *J. Exper. Med.* 85: 2, 163-173, 1947.
8. Rammelkamp, C. H.: *A Method for Determining the Concentration of Penicillin in Body Fluids and Exudates*, *Proc. Soc. Exper. Biol. & Med.* 51: 95, 1942.



# THE ANTIBACTERIAL ACTIVITY OF SOME SULFON- AND SULFANILANILIDE DERIVATIVES

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RENSSELAER, N. Y.

AN EARLIER communication<sup>1</sup> from these laboratories presented the general antibacterial activity of a series of benzenesulfonic acid derivatives. The most effective compound there referred to was found to be 3',5'-dibromosulfanilamide or the corresponding dichloro derivative. The removal of the amino group resulted in only a slight loss in activity. Several monohalogenated and dihalogenated compounds were tested in which the bromine or chlorine atoms were placed in various positions on the anilide portion of the molecule. Antibacterial activity was practically lost when a halogen was placed in the 2-position. While all these compounds displayed definite antibacterial action, none approached that of 3',5'-dibromosulfanilamide. The latter compound was also tested for antibacterial properties against pathogens, most of which cause infections which do not readily respond to present chemotherapeutic agents.<sup>2</sup>

In order to obtain a better correlation between chemical structure and degree of antibacterial action, it seemed worth while to prepare additional benzenesulfonic acid derivatives. Accordingly, the following compounds\* were tested by the present authors.

COMPOUND	NAME	MOL. WEIGHT
1	2',6' dichlorosulfanilamide	302.2
2	3',5'-dibromo 4'-hydroxybenzenesulfonamide	407.0
3	3',5'-dibromo 4'-hydroxysulfanilamide	422.1
4	3' bromo 5' chloro-4' hydroxysulfanilamide	377.6
5	3' bromo 5' chlorosulfanilamide	361.6
6	3',5' diodo 4' hydroxysulfanilamide	516.0
7	3',5'-diiodosulfanilamide	500.0
8	3',5'-bis-(trifluoromethyl) sulfanilamide	384.3
9	3' trifluoromethylsulfanilamide	316.3
10	3',5' dibromo o aminobenzenesulfonamide	406.1
11	3',5' dibromo m aminobenzenesulfonamide	406.1
12	N <sup>4</sup> succinyl 3',5' dibromosulfanilamide	506.2
13	3',5' dibromosulfanilamide	406.1

## METHODS

The method for testing the antibacterial action of the compounds was essentially the same as that outlined in the previous report.<sup>1</sup> This consisted of dissolving the drugs in a few c.c. of alcohol preparatory to making an initial dilution in nutrient broth. Serial dilutions were prepared in broth from 1:1,000 up to and including 1:1,024,000. After autoclaving, the drug broth mixtures were inoculated with a test organism and incubated at 37° C. for seventy two hours. Tubes containing no growth or growth less than one half that of the drug-free control after incubation for twenty four hours (forty eight hours in test with *Actinomyces bovis*) were considered to contain a bacteriostatic concentration of drug. All

From the laboratories of the Winthrop Chemical Co., Inc

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\*Prepared by Dr. A. Mooradian of the Sterling Winthrop Research Institute

TABLE 1. HIGHEST DILUTION OF C

COM- POUND	PNEUMOCOCCUS TYPE I		STREPTOCOCCUS PYOGENES-C203		STREPTOCOCCUS FAECALIS		STREPTOCOCCUS VIRIDANS		STAPHYLOCOCCUS AUREUS		NEISSERIA GONORRHOEA	
	BS*	BC	BS	BC	BS	BC	BS	BC	BS	BC	BS	BC
1	2	<1	<1	<1	-	-	-	-	2	<1	-	-
2	8	4	4	2	4	<1	4	1	32	1	16	8
3	32	8	2	2	16	<1	2	<1	4	<1	8	16
4	32	16	4	2	16	<1	4	<1	8	<1	16	1
5	128	64	64	32	16	<1	16	8	128	1	32	3
6	32	16	4	2	8	<1	4	<1	16	<1	8	6
7	64	64	32	16	32	<1	32	<1	128	<1	256	6
8	256	128	64	16	16	<1	16	16	512	128	64	6
9	64	32	64	16	4	2	8	4	16	4	32	12
10	32	2	128	<1	<1	1<	<1	<1	128	<1	256	1
11	64	32	16	16	8	<1	16	<1	512	<1	32	3
12	4	2	4	2	1	<1	2	1	32	1	8	3
13	128	64	32	32	<1	1<	16	8	64	<1	64	3

\*Figures represent dilutions bacteriostatic (Bs) or bactericidal (Bc) as expressed in thousands, is, 128 = 128,000.

<1, Concentrations greater than 1:1,000 not tested; —, not tested.

tubes in which growth failed to appear after incubation for seventy-two hours were subcultured into fresh drug-free broth, and the subculture tubes incubated for an additional seventy-two hours. Failure of growth to develop in the latter was taken as evidence of a bactericidal action in the tubes from which they were subcultured. Bacteriostatic and bactericidal action against the gonococcus was determined by subculturing on dextrose-starch agar.\* In the study, 3',5'-dibromosulfanililide (Compound 13) was included for purpose of comparison. The results of this investigation are presented in Table I.

## RESULTS

The only compound which appears to approach or to exceed the activity of 3',5'-dibromosulfanililide (compound 13) is a derivative containing a large number of fluorine radicals (compound 8). The iodo analogue (compound 7) of compound 13 and the 5-bromo-3-chloro derivative (compound 5) appear to exert an activity comparable to that of the parent compound. The introduction of the hydroxyl group on the 4-position of the anilide invariably results in a decrease of activity (compare compound 3 with 13, 4 with 5, and 6 with 7). The succinyl derivative (compound 12) also displays a marked decrease in antibacterial action.

The ortho- and meta-isomers (compounds 10 and 11) present an action equal to that of 3',5'-dibromosulfanililide when all the test organisms are collectively taken into consideration. This would tend to indicate that a free amino group in the para-position is not essential for maximum activity, and may thus explain why the action of 3',5'-dibromosulfanililide against *Streptococcus pyogenes* is unaffected by the presence of p-aminobenzoic acid.

## SUMMARY

Several new derivatives of 3',5'-dibromosulfanililide have been prepared and tested for antibacterial action in vitro against type I pneumococcus, *Streptococcus pyogenes* (C203), *Streptococcus faecalis*, *Streptococcus viridans*,

\*Difco Laboratories, Inc., Detroit, Mich.

UNDS EXHIBITING ANTIBACTERIAL ACTION

BRUCELLA ABORTUS		HEMOPHILUS DUCREYI		PASTEURILLA PESTIS		VIBRIO CHOLERA		CLOSTRIDIUM WELCHII		ACTINOMYCES BOVIS	
BS	BC	BS	BC	BS	BC	BS	BC	BS	BC	BS	BC
32	<1	-	-	-	-	-	-	<1	<1	-	-
8	2	16	1	32	2	16	2	16	2	64	2
32	1	16	1	32	2	64	1	1	1	16	1
8	1	16	<1	64	2	32	2	1	1	1	<1
16	8	16	8	128	16	32	8	8	2	128	16
1	<1	16	1	16	2	32	8	8	2	16	1
64	<1	64	16	128	<1	32	<1	64	2	256	16
128	<1	32	<1	32	<1	32	<1	64	32	128	32
32	4	16	2	32	1	16	1	8	4	32	8
128	<1	<1	<1	2	<1	1	<1	1	1	256	64
64	<1	8	<1	64	1	32	<1	16	16	64	32
8	1	2	<1	8	1	8	1	2	1	8	1
64	16	512	32	64	2	32	1	32	16	128	16

*Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Clostridium welchii*, *Brucella abortus*, *Hemophilus ducreyi*, *Pasteurella pestis*, *Vibrio cholerae*, and *Actinomyces bovis* (anaerobic).

The 3',5'-bis-(trifluoromethyl) derivative was found to be somewhat superior to 3',5'-dibromosulfanililide, while the 3',5'-diiodo and the 3'-bromo-5'-chloro derivatives were about equal to 3',5'-dibromosulfanililide in antibacterial action.

The ortho- and meta isomers in which the NH<sub>2</sub> group is not in the para-position display an activity equal to 3',5'-dibromosulfanililide, while the hydroxysulfanililide and succinyl derivatives which were tested were found to be less active than the latter compound

Since this paper was prepared, Schmidt and Sesler<sup>3</sup> demonstrated that 3',5' dibromo sulfanililide and a series of derivatives therefrom held little promise as effective chemotherapeutic agents in bacterial infections. McChesney<sup>4</sup> more recently described the toxicity, absorption, and metabolism of 3',5' dibromosulfanililide which account for its relative ineffectiveness in infection protection tests in experimental animals

# REFERENCES

- Goetchi, G. R., and Lawrence, C. A.: A Series of New Sulfonamides Which Are Unaffected by p Aminobenzoic Acid, *J. Bact.* 49: 575-584, 1945.
- Goetchi, G. R., and Lawrence, C. A.: Further Studies on the Antibacterial Action of 3',5' Dibromosulfanililide, *J. LAB. & CLIN. MED.* 31: 336-339, 1946.
- Schmidt, L. H., and Sesler, C. L.: On the Chemotherapeutic Properties of the Sulfanililides, *J. Pharmacol. & Exper. Therap.* 87: 313-328, 1946.
- McChesney, E. W.: 3',5' Dibromosulfanililide. Toxicity, Absorption, and Metabolism, *J. Pharmacol. & Exper. Therap.* 89: 368-378, 1947.

# THE EFFECT OF 2,3-DIMERCAPTOPROPANOL (BAL) ON THE TOXICITY OF GOLD

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**B**AL (2,3-dimercaptopropanol) was first introduced as an agent capable of combating the arsenic toxicity which results from Lewisite burns.<sup>1</sup> In addition to reducing the toxicity of compounds of arsenic,<sup>2</sup> this sulfhydryl compound has been shown to diminish the toxicity of antimony,<sup>3</sup> mercury,<sup>3</sup> chromium,<sup>3</sup> bismuth,<sup>3</sup> and nickel.<sup>4</sup>

Gold compounds have found rather widespread clinical use in the treatment of rheumatoid arthritis. However, due to the toxicity of this heavy metal, extreme caution is required in its therapeutic use. The purpose of this study was to determine in experimental animals whether some of the gold compounds commonly used as therapeutic agents could be rendered less toxic by the use of BAL.

While this work was in progress there appeared a series of papers covering the effect of BAL on the toxic symptoms produced by gold in human subjects.<sup>5-7</sup> Ragan and Boots<sup>8</sup> mentioned that BAL reduces the toxicity of gold in rats, but few data were presented.

## METHODS

For acute toxicity studies, groups of adult hooded rats, varying in weight from 190 to 326 grams, were given one of the gold compounds studied. All administrations were made intramuscularly and the survival time was recorded. The dose of the gold compound administered was that which was determined by preliminary trial to cause death within a few days. One control group of animals received only the gold compound. A second (test) group received the same dose of the same gold compound in one thigh and BAL in the muscle of the opposite leg. Additional groups of rats received the same dose of gold, but the BAL was administered in multiple doses. Any change in the survival time was considered to be due to the modification of the toxicity of the gold compound tested.

In the studies of the effect of gold and BAL on the rate of growth of young rats, all the animals except those which received neither gold nor BAL were litter mates. In this portion of the experiment the animals received a single sublethal dose of gold and also a single dose of BAL. Weights usually were recorded every second day during the early portion of this work; later a longer time interval was used. Growth was recorded for a period of twenty-three days. At the end of this period the animals were sacrificed and portions of the liver and kidney were fixed in formalin for microscopic examination.

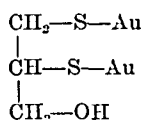
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The toxicities of the following gold-containing compounds were studied: gold sodium thiosulfate, gold sodium thiomalate (Myoechrysine\*), sodium succimidoaurate,† and gold thioglucose.‡ The last substance was supplied both as a pure solid and as a suspension in an oily medium. A commercial 10 per cent solution of BAL in oil and benzyl benzoate was used.

It was found that if an aqueous solution of BAL was added to an aqueous unbuffered solution of either gold sodium thiosulfate or gold sodium thiomalate a heavy yellow precipitate formed immediately. In the case of sodium succimidoaurate and gold thioglucose no precipitate formed on mixing solutions of these compounds with aqueous BAL. The BAL-gold compound proved to be insoluble in water, dilute HCl or NaOH, alcohol, and ether. The BAL-gold compound formed with either gold sodium thiomalate or gold sodium thiosulfate was washed successively with water, alcohol, and ether, and dried in a vacuum dessicator over sulfuric acid. The content of gold in each sample was determined by ashing according to the method of Pregl<sup>5</sup> and by weighing the gold residue. In addition, a sample of the BAL-gold compound containing two atoms of gold per molecule of BAL was prepared according to the procedure of Weiss<sup>6</sup> by adding one mol of gold bromide to 0.8 mol of BAL. The analyses of the compounds are given below:

BAL-gold compound from gold sodium thiosulfate	72.6 per cent Au
BAL-gold compound from gold sodium thiomalate	73.7 per cent Au
BAL-gold compound from gold bromide	73.6 per cent Au
Theoretical $C_3H_6O_2SAu_2$	76.4 per cent Au

These analyses indicate that the compounds formed are about 95 to 96.5 per cent pure. The probable structure for the BAL-gold compounds is:



## RESULTS

Gold sodium thiosulfate was found to be a toxic compound. Six groups of animals were studied. The results of this experiment are summarized in Table I. Rats given this compound in doses equivalent to 75 mg. of gold per kilogram usually died within six hours. The administration of 12.5 mg. of BAL just before the gold sodium thiosulfate appeared to reduce the toxicity of the gold compound by about one-third. The survival time of the rats was the same whether a single dose of BAL was given either one minute before or one hour after the gold. If two doses of BAL were given, one a minute after the gold and the second five hours later, there was a prolongation of the survival time as compared with a single dose of BAL. Four of the ten animals in this series survived the test period of fourteen days and then were sacrificed. If three doses of BAL were administered at 1-minute, 3-hour, and 6-hour intervals after the gold, the effects were in general similar to those obtained with two doses of BAL. However, only two of the animals in this group survived the period of study. The animals which survived only about six hours probably died of respiratory and vasomotor paralysis.<sup>10</sup> The animals which lived three to six

\*Merck & Co., Inc., Rahway, N. J.

†This compound was supplied through the courtesy of Inventions, Inc., Chicago, Ill.

‡Both Solganal-B Oleosum (gold thioglucose) and the pure gold thioglucose were supplied by the Schering Corporation, Bloomfield, N. J.

TABLE 1. EFFECT OF BAL ON THE TOXICITY OF GOLD COMPOUNDS; BOTH BAL AND GOLD COMPOUNDS ADMINISTERED SUBCUTANEOUSLY

SUBSTANCE	DOSE OF GOLD (MG./ KG.)	BAL. ADMINISTERED		SURVIVAL TIME (DAYS)
		MG.	TIME	
			BEFORE Au	
Gold sodium thiosulfate	30	0		2,2,2,3
	75	0		1,0,1/4,1,1,1/4,1/4,1/4,1/4,1/2,1/2
	75	12.5	1 min.	2,2,2,3,4,4
	75	12.5	1 hr.	3,3,3,4
	75	12.5	1 min.	
		12.5	5 hr.	2,3,5,5,5,6, s, *s,s,s
	75	12.5	1 min.	
		12.5	3 hr.	
		12.5	6 hr.	3,3,3,4,4,4,6, s,s
Gold sodium thiomalate	75	0		3,4,4,5,6,6, s,s
	75	12.5	1 min.	3,3,4,5,5,5
	100	0		3,3,4,4,4,4
	100	12.5	5 min.	2,2,4,5,5,7
	100	12.5	3 hr.	
		12.5	24 hr.	3,4,5,5
	100	12.5	1 min.	
		12.5	4 hr.	
Gold thioglucose		12.5	7 hr.	2,2,2,3,4,5
	75	0		s,s,s,s,s,s
	100	0		3,3,4,4,4
	100	12.5	1 min.	2,3,4,6,8,9
	100	12.5	1 min.	
		12.5	3 hr.	
Sodium succimidoaurate		12.5	6 hr.	s,s,s,s,s,s,s
	75	0		3,3,3,3,4,4
	75	12.5	1 min.	s,s,s,s,s,s

\*s. The animal survived for the experimental period of fourteen days.

days showed large soft, grayish spotted kidneys on death. Microscopic examination of the kidney showed extreme damage to the tubules of this organ. Renal insufficiency was believed to be the cause of death.

In the study of acute toxicity, gold sodium thiomalate was administered to six groups of animals. The results of these experiments also are summarized in Table I. The amount of gold used in the first group of animals in this series was equivalent to that used in the gold sodium thiosulfate study (75 mg. per kilogram). Survival time for the animals treated with gold sodium thiomalate was considerably longer than that observed with gold sodium thiosulfate. The administration of BAL immediately after giving gold sodium thiomalate did not appear to have any effect on the survival time of the animals. In the remainder of this study, the dose of gold sodium thiomalate was increased to the equivalent of 100 mg. of gold per kilogram in order to decrease the survival time of the animals. Thus, any detoxifying effect of BAL could be more easily detected. The administration of 12.5 mg. of BAL to animals five minutes after the dose of gold appeared to have no effect on the survival time. If two doses of BAL were given, one three hours and the other twenty-four hours following the gold, there was no prolongation of the survival time as compared with the control animals of this series. If three doses of BAL were given, one immediately after the gold, the second four hours later, and the last seven hours after the gold, there was a slight decrease in the length of their survival. This effect may have been due to the sum of the toxicities of BAL and gold.

The toxicity of sodium succinimidoaurate can be effectively reduced by the administration of BAL after the gold compound. Table I shows that, following administration of the BAL, there were no deaths due to gold.

An attempt was made to produce death in rats by administering gold thioglucose which was supplied in an oil media, but the dose required to produce death consistently was too high to have value in this study. Four of the rats received as much as 2 ml. of the oily suspension which contained 200 mg. of gold (approximately 800 mg. per kilogram). Two of the animals survived and two died, one on the third and one on the fifth day after injection. At autopsy, the animal that lived only three days had a large mass of yellow oil at the site of injection, showing that the absorption of the gold compound was poor. On the other hand, if an aqueous solution of gold thioglucose was administered in a dose supplying 100 mg. of gold per kilogram, the animals usually died within four days. Three doses of BAL completely protected the animals, as shown by survival of all seven animals tested.

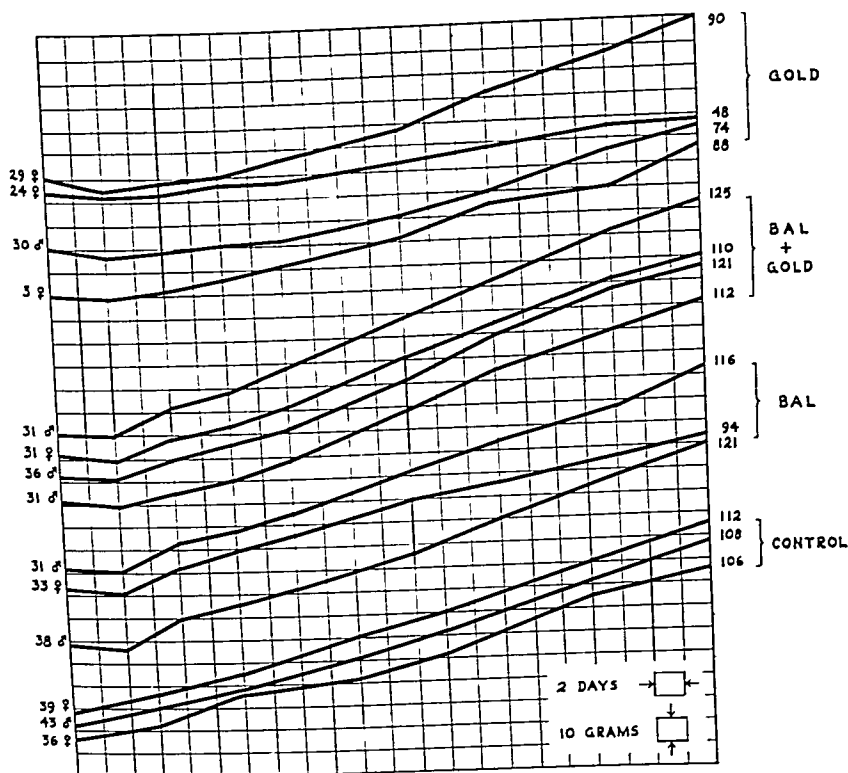


Fig. 1.—The effect of a single dose of gold sodium thiomalate on the growth of young rats. The dose of gold was 50 mg. per kilogram and that of BAL 3.1 mg. per animal. These compounds were given intramuscularly, at the start of the experiment. The numbers along the left border are the initial weight in grams and those on the right are the final weight twenty-three days later.

The effect of BAL on the rate of growth of rats given a nonlethal dose of gold (50 mg. per kilogram) was considered a more sensitive test than the survival time. The young rats which received the single dose of gold sodium thiomalate had rather shallow growth curves, while those which received the

same dose of gold plus BAL (3.1 mg.) had growth curves of approximately the same slope as those which received no treatment or only BAL (Fig. 1). Microscopic examination of the livers and kidneys of these animals was made. The kidneys of those animals which received only the gold showed "marked cloudy swelling of the proximal convoluted tubules with karyolytic and karyorrhectic nuclear changes and plugging of the tubules with necrotic debris some of which contains calcium. There are evidences of regenerative epithelial hyperplasia of the cells of these tubules. There is marked cloudy swelling and nuclear degeneration of scattered liver cells."<sup>6</sup> Those which received the gold followed by the BAL showed "moderate cloudy swelling of the cells of the proximal convoluted tubules. A few basophilic hyaline casts are seen."<sup>6</sup> BAL alone produced a similar picture.

#### DISCUSSION

In this study of the effect of BAL on the toxicity of various gold compounds, it is of interest that the gold compounds were of different chemical classes. The gold sodium thiosulfate contained monovalent gold bound in an inorganic linkage. Gold sodium thioglucose and gold sodium thiomalate contained monovalent gold bound to an organic radical. The attachment of the gold to the carbon chain was through a sulfur linkage. Sodium succimidoaurate contained trivalent gold bound in a sulfur-free organic compound.

The data presented in Table I show that, under the conditions described, BAL reduces the toxicity of gold with all compounds tested except gold sodium thiomalate. It should be noted that the doses of gold used in the acute toxicity studies were relatively high. If smaller doses of gold sodium thiomalate were given and growth (as measured by an increase in weight) was used as a criterion of toxicity, BAL effectively detoxified gold sodium thiomalate.

When BAL was given with large doses of gold sodium thiosulfate the animals usually survived the factors which produced rapid death, but some of them later succumbed to the nephrotoxic effect of the heavy metal. This action also has been noted where the effect of BAL on cadmium poisoning was studied.<sup>4</sup> The explanation given was that the BAL-metal compound is reabsorbed by the kidney tubule and produces toxic action there.

#### SUMMARY

The effect of BAL on the survival time of rats given subcutaneous injections of lethal doses of gold, as gold sodium thiosulfate, gold sodium thiomalate, gold thioglucose, or sodium succimidoaurate was studied. BAL caused an increase in survival time with all compounds studied except gold sodium thiomalate. With sublethal doses of gold sodium thiomalate, BAL has a definite effect in reducing toxicity of this heavy metal when growth was used as a criterion.

#### REFERENCES

1. Waters, L. L., and Stock, C.: BAL (British Anti-Lewisite), *Science* 102: 601, 1945.
2. Eagle, H., Magnuson, H. J., and Fleishman, R. J.: Clinical Uses of 2,3-Dimercaptopropanol (BAL). I. The Systemic Treatment of Experimental Arsenic Poisoning (Mapharsen, Lewisite, Phenylarsenoxide) with BAL, *J. Clin. Investigation* 25: 451, 1946.

\*The interpretations of the tissue changes in this study were by Dr. J. L. Chason of the Department of Pathology of the Wayne County General Hospital, Eloise, Mich.



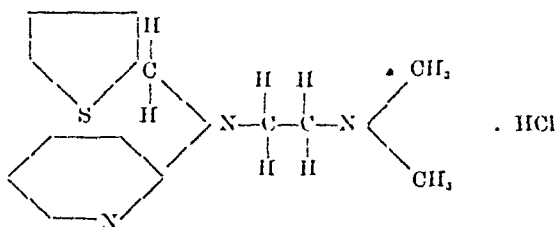
3. Braun, H. A., Lusk, L. M., and Calvery, H. O.: The Efficiency of 2,3-Dimercaptopropanol (BAL) in the Therapy of Poison by Compounds of Antimony, Bismuth, Supplementary Chromium, Mercury and Nickel, *J. Pharmacol. & Exper. Therap.* 87: 119, 1946.
4. Gilman, A., Philips, F. S., Allen, R. P., and Koelle, E. S.: Treatment of Acute Cadmium Intoxication in Rabbits with 2,3-Dimercaptopropanol (BAL) and Other Mercaptans, *J. Pharmacol. & Exper. Therap.* 87: (Supp.) S5, 1946.
5. Cohen, A., Goldman, J., and Dubbs, A. W.: Gold and Arsenic Poisoning, *J. A. M. A.* 133: 749, 1947.
6. Ragan, C., and Boots, R. H.: Gold Dermatitis, *J. A. M. A.* 133: 752, 1947.
7. Lockie, M., Norcross, B. M., and George, C. W.: Reactions Due to Gold, *J. A. M. A.* 133: 754, 1947.
8. Roth, Hubert: *Pregl's Quantitative Organic Microanalysis*; translated by E. Beryl Daw, Philadelphia, 1937, P. Blakiston's Son & Co.
9. Weiss, U.:  $\alpha$ -Auromercaptoacetanilide, *J. Am. Chem. Soc.* 67: 1424, 1945.
10. Schlozmann, H.: Gold in Heffter, A., *Handbuch der Experimentelle Pharmatologie*, Vol. 3, Part 3, Berlin, 1934, Julius Springer, p. 2118.

## HISTAMINE ANTAGONISTS

### VIII. N-( $\alpha$ -PYRIDYL)-N-( $\alpha$ -THIENYL)-N',N-DIMETHYLETHYLENEDIAMINE, A NEW ANTIHISTAMINIC COMPOUND. EXPERIMENTAL AND CLINICAL EXPERIENCES

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A NEW antihistaminic drug of the ethylenediamine series, N-( $\alpha$ -pyridyl)-N-( $\alpha$ -thienyl)-N',N-dimethylethylenediamine hydrochloride, made available to us about a year ago,<sup>\*</sup> has been found to possess potent clinical and pharmacologic activities. This substance differs from pyribenzamine in the substitution of a thienyl for the benzyl ring and has the following structural formula<sup>†</sup>:



#### EXPERIMENTAL FINDINGS

This substance is similar to most of the other antihistaminic drugs in its local anesthetic action. It was found to be a potent antihistaminic substance. Death in guinea pigs from an intravenous M.L.D. 100 dose of histamine† (0.4 mg. histamine base per kilogram) could be prevented in 100 per cent of the animals by the intraperitoneal injection thirty minutes previously of 0.1 mg. of the drug per kilogram. A dose of 0.05 mg. per kilogram protected 50 per cent of the animals. Roth and associates<sup>2</sup> found that 5 mg. of the drug per kilogram intraperitoneally was effective in protecting guinea pigs against 60 M.L.D. 100 of histamine, while Ereoli and associates<sup>3</sup> obtained protection against 150 to 200 lethal doses of histamine by using 10 to 15 mg. of the drug per kilogram.

Roth and associates report that 0.002  $\gamma$  per cubic centimeter produces 75 per cent or greater inhibition of the contraction of the guinea pig ileum from 0.005  $\gamma$  per cubic centimeter of histamine base. The activity of this drug in the inhibition of the ileal contraction, as compared with other drugs, is esti-

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\*The drug was submitted to us by the research departments of Eli Lilly & Co., Indianapolis, Ind. (Histadyl), and of Abbott Laboratories, North Chicago, Ill. (Thienylene), without either firm being cognizant until several months later that both had synthesized the same compound.

†Histamine diphosphate supplied through the courtesy of Burroughs Wellcome & Co., Inc., New York, N. Y.

mated by Lee and associates<sup>4</sup> as follows: benadryl, 1; pyribenzamine, 4.9; and the thienyl compound, 3.9. Such active inhibition also was found by Ercoli and associates<sup>3</sup> and by us.

Protection against death from aerosolized histamine was studied by Lee and associates, who found the drug six times more potent than benadryl and less than half as potent as pyribenzamine. Ercoli and associates<sup>3</sup> also reported effective protection against histamine aerosol.

Our work along this line was somewhat different. Because of our special interest in the possible therapeutic value of aerosols of antihistamine drugs in asthma, we wished to study the effect of such drug aerosols as protection against bronchospasm from aerosolized histamine. In order to approximate conditions more closely resembling those in human asthma we selected unquestioned labored breathing, rather than death, as the end point in the guinea pig. The animals were exposed for five minutes to a standard aerosol of the drug in one chamber, and after a period of fifteen minutes they were exposed to the standard histamine aerosol. The time elapsing before the onset of dyspnea was noted. By varying the concentrations of the drug aerosol on different days in the same series of animals a fair estimate of the protective ability of the drug could be made. Many experiments with many variations were performed and comparative studies of all available drugs were made. The complete details will be reported at another time. Here we wish to note briefly that moderate concentrations of the drug aerosol ( $\frac{1}{2}$  to 2 per cent), for five minutes at 5 pounds pressure with a particular atomizer were effective in giving appreciable protection against histamine aerosol ( $\frac{1}{2}$  mg. of base per cubic centimeter at 5 pounds pressure). This protection was not quite as good as that obtained with pyribenzamine, but better than that obtained with benadryl.

The intraperitoneal injection of 1 mg. per kilogram of this drug in guinea pigs was effective in protecting ten of ten animals against anaphylactic death, while a large control series showed a mortality of 61 per cent.

#### CLINICAL FINDINGS

The thienyl compound has been found by us to be an effective remedy in the symptomatic relief of allergic manifestations. In 112 patients with seasonal hay fever (including that due to the pollen of trees, grasses, and weeds, and to the spores of molds) seventy-nine, or 70 per cent, were benefited. Of ninety-five patients with nonseasonal vasomotor rhinitis, forty-four, or 46 per cent, received some measure of relief. The dyspnea of asthma was not appreciably altered in thirty patients, although the preasthmatic, spasmodic cough was decidedly helped in six of nine patients. The subjective symptoms of urticaria were helped in seven of twelve patients. In thirteen patients with atopic dermatitis eight obtained considerable relief from the itching. Seven of nine patients with dermatographism and one with pruritus of unknown origin were benefited. Pruritus ani was substantially relieved in two of three patients. The experimental wheal produced by histamine or by a specific antigen on the human skin was diminished by the local application of the drug.

As with other previously described antiallergic drugs with antihistaminic action,<sup>5, 6</sup> the relief was only palliative, lasting two to six hours, was not complete, since all manifestations of the allergic ailment were not abolished, and was not obtained in all allergic patients. From our experience thus far, we gain the impression that this drug is an effective agent in the alleviation of allergic manifestations, particularly allergic rhinitis and itching dermatoses. Although the incidence, and perhaps the degree, of relief is not quite as high as that obtained with pyribenzamine, the thienyl compound has the common attribute of other antihistaminic compounds in its selective superiority for particular individuals. In other words, in some patients this drug is more effective than other drugs with a higher therapeutic index.

The average dose of the drug is 50 mg. orally, one to four times daily, depending on the nature of the manifestations. In a few patients we employed 100 mg. doses, but these were frequently not well tolerated. In 253 patients, most of whom received 50 mg. doses, undesirable side actions were noted in sixty-three, or 25 per cent. Sedation was the most common finding, occurring in forty-eight, or 19 per cent, of the patients. The degree of sedation was not as great as that produced by benadryl, but approximated or perhaps exceeded that experienced with pyribenzamine. Our limited use of 100 mg. doses gives us the impression that marked sedation is probably more common than with similar doses of pyribenzamine. Here again, as with therapeutic individuality, we found individuality in sedative effects. We encountered patients who experienced sedation from pyribenzamine and no sedation from the thienyl compound. Other but less frequent side actions noted were vertigo, nervousness, dryness of the mouth and throat, excitation, insomnia, headache, nausea, and diarrhea.

#### SUMMARY

1. A new antihistaminic compound, N-( $\alpha$ -pyridyl)-N-( $\alpha$ -thienyl)-N',N'-dimethylethylenediamine hydrochloride, is a potent agent in the prevention of death from histamine injection or aerosol in the guinea pig, in the inhibition of the histamine contraction of the guinea pig ileum, and in the prevention of anaphylactic shock.

2. Aerosols of this drug are capable of preventing bronchospasm and dyspnea in guinea pigs from aerosolized histamine.

3. This drug has a high incidence of effectiveness in the symptomatic relief of some allergic conditions, particularly allergic rhinitis (seasonal and nonseasonal), urticaria, and atopic dermatitis.

4. Side reactions are fairly common and consist mainly of sedation. In degree and incidence sedation is less than that obtained with benadryl and equals or exceeds that of pyribenzamine.

5. The general experimental and clinical efficacy of this drug approaches but does not equal that of pyribenzamine. However, its availability is an advantage because in selected persons it is more effective or less disturbing than pyribenzamine.

6. Neither this nor any other antihistaminic drug has any but palliative function and does not replace the more basic and more lasting benefit obtained from allergic management by avoidance or desensitization.

## REFERENCES

1. Weston, A. W.: N, N-Dimethyl-N'-( $\alpha$ -Pyridyl)-N'-( $\alpha$ -Thienyl)-Ethylenediamine, an Anti-histaminic Agent, *J. Am. Chem. Soc.* 69: 980, 1947.
2. Roth, L. W., Richards, R. K., and Shepperd, I. M.: Some Pharmacologic Properties of a New Anti-Histamine Compound, *Federation Proc.* 6: 366, 1947.
3. Ereoli, N., Schachter, R. J., Leonard, F., and Salmssen, V. V.: A New Antihistaminic, N, N-Dimethyl-N'-(2 Pyridyl)-N'-(2 Thienylmethyl)-Ethylenediamine Hydrochloride —W53, *Arch. Biochem.* 13: 487, 1947.
4. Lee, H. M., Dinwiddie, W. G., and Chen, K. K.: The Antihistamine Action of N-(2-Pyridyl)-N-(2 Thenyl)-N', N'-Dimethylethylenediamine Hydrochloride, *J. Pharmacol. & Exper. Therap.* 90: 83, 1947.
5. Feinberg, S. M.: Report to the Council on Pharmacy and Chemistry. Histamine and Antihistaminic Agents, Their Experimental and Therapeutic Status, *J. A. M. A.* 132: 702, 1946.
6. Feinberg, S. M.: *Allergy in Practice*, ed. 2, Chicago, 1946, The Year Book Publishers, Inc., Page 801.

# EFFECT OF ALKALOIDS ON ACETYLCHOLINE AND POTASSIUM SENSITIVITY OF STRIATED MUSCLE

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**I**N A study dealing with the mechanism through which muscle shortening is induced, data about agents that modify the induction of muscle shortening may be essential.

In the following, the effects of various concentrations of different alkaloids on the shortening of striated muscle induced by acetylcholine and potassium were investigated.

## EXPERIMENTAL

### *Effect of Alkaloids on Acetylcholine Sensitivity of Striated Muscle.—*

**Method:** The rectus abdominis muscle of the frog was excised and suspended in a muscle chamber containing 10 c.c. frog Ringer's solution. Shortening of the muscle was induced throughout the experimental procedure by immersion in Ringer's solution containing acetylcholine bromine (50  $\mu$ g per 100 c.c.) for two minutes. Between two immersions in acetylcholine the muscle was washed with Ringer's solution for ten minutes. This procedure was repeated until three successive immersions in the solution of acetylcholine gave similar responses. The shortening of muscle was registered by an isotonic lever on a kymograph. After this stabilization, between two shortenings induced by the acetylcholine solution, instead of washing with Ringer's solution for ten minutes, the muscle was washed for five minutes and was immersed for five minutes in Ringer's solution containing one of the alkaloids to be investigated. Solutions containing the alkaloids in increasing concentrations were used. The pH of all solutions was corrected to 7.

**Control:** Muscles were immersed only in Ringer's solution and shortening was induced with the acetylcholine solution as described. The repeatedly induced shortenings, each lasting two minutes, were of the same magnitude for at least three hours. This period of time was longer than the duration of the experiments described.

**Calculation:** The amount of shortening of muscle after immersion in the various solutions to be tested was expressed as a percentage of the amount of shortening of the same muscle induced by acetylcholine before immersion in the solution of alkaloids. All results deviating from 100 per cent by more than twice the square root of the sum of the squares of the standard error of mean of the controls and the standard error of mean of the experiments were considered significant [ $2 \sqrt{S.E.^2(\text{control}) + S.E.^2(\text{experiments})}$ ].

**Results:** The effects of the alkaloids on the shortening of the rectus abdominis muscle induced by acetylcholine are given in Table I. The muscle shortening increased in the presence of physostigmine, colchicine, epinephrine, ergotamine, pilocarpine, morphine, and yohimbine (the latter three in higher concentrations). The other alkaloids used decreased the acetylcholine sensitivity of the muscle.

**Effect of Alkaloids on Potassium Sensitivity of Striated Muscle.—**In the following experiments the effect of the alkaloids on the shortening of muscle

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induced by potassium was investigated to ascertain whether the alkaloids modify the sensitivity of muscle to chemical stimuli other than acetylcholine.

*Method:* Shortening of muscle was induced by a 20 mM KCl solution instead of acetylcholine, and the effect of the substances on the potassium sensitivity of the muscle was observed as described previously.

*Results:* The results are given in Table I. The shortening of muscle induced by potassium increased in the presence of all alkaloids (except epinephrine and cocaine), veratrine and colchicine being the most effective agents. The potassium sensitivity decreased in the presence of epinephrine and cocaine and in the presence of higher concentrations of d-tubocurarine, strychnine, quinine, quinidine, and atropine.

TABLE I. EFFECT OF ALKALOIDS ON THE SHORTENING OF THE RECTUS ABDOMINIS MUSCLE INDUCED BY ACETYLCHOLINE AND POTASSIUM

SUBSTANCE	AMOUNT OF CONTRACTION (IN PER CENT OF CONTROL <sup>*</sup> ); SHORTENING INDUCED WITH											
	ACETYLCHOLINE						POTASSIUM					
	CONCENTRATIONS OF THE SUBSTANCES USED (MG. PER 100 C.C. RINGER'S SOLUTION)											
	100	10	1	0.1	0.01	0.001	100	10	1	0.1	0.01	0.001
Atropine	24	34	88	107	108	107	79	114	109	113	107	103
Amphetamine	23	74	92	98	99	98	88	108	110	134	132	122
Cinchonine	---	11	79	96	94	102	---	117	116	112	113	99
Cocaine	---	s†	77	97	101	102	---	s	77	85	90	96
Codeine	56	77	91	94	99	100	149	149	143	144	137	---
Colchicine	---	s	153	138	123	100	---	s	445	224	140	133
Ephedrine	67	96	100	101	102	100	130	117	135	132	116	112
Epinephrine	---	---	137	116	98	101	---	---	58	66	83	---
Ergotamine	---	174	117	110	110	105	---	291	170	155	145	123
Morphine	130	99	98	98	99	100	138	144	138	139	134	128
Physostigmine	---	---	620	295	130	100	153	132	135	131	130	100
Pilocarpine	198	140	105	104	103	102	126	137	149	133	129	107
Quinidine	s	21	79	82	89	102	s	71	98	124	119	110
Quinine	s	6	34	83	95	97	s	86	105	136	127	122
Strychnine	s	10	13	44	83	103	s	67	78	107	104	117
d-Tubocurarine	---	5	9	34	76	98	---	73	88	93	105	114
Veratrine	---	s	58	109	107	110	---	s	720	510	237	178
Yohimbine	s	116	96	99	95	99	s	110	108	101	110	106

\*Each value represents the average of ten separate experiments. The S. E. of the mean for each value was less than  $\pm 4$  per cent.

†S, Shortening of the muscle occurred during the immersion of the muscle in the solution for five minutes, without addition of acetylcholine or potassium.

## DISCUSSION

Shortening of muscle induced by acetylcholine and by potassium involves mechanisms not identical in all details. Acetylcholine and potassium induce different physicochemical changes in the muscle cells<sup>2-6</sup> and even different physical changes in isolated myosin. Acetylcholine sensitivity parallels in many respects the sensitivity of muscle to indirect stimulation, whereas potassium sensitivity often parallels the effect of direct stimulation.<sup>7</sup>

The alkaloids used can be divided into two groups according to their effect on acetylcholine sensitivity: alkaloids increasing the acetylcholine sensitivity of striated muscle and alkaloids with a curarelike effect of decreasing the acetylcholine sensitivity.

The acetylcholine sensitivity of striated muscle was increased by at least two mechanisms: decrease of the activity of cholinesterase<sup>1,17</sup> and direct effect on the striated muscle. That alkaloids exerted a direct effect on the muscle cell may be concluded since changes of acetylcholine sensitivity often did not parallel the degree of inhibition of the activity of cholinesterase,<sup>1,17</sup> and the alkaloids modified the potassium sensitivity of the muscle.

The decreased potassium sensitivity of muscle immersed in epinephrine was, at least partly, due to the fact that epinephrine reduces the potassium content of the muscle cell (as documented by Torda and Wolff<sup>1</sup>). Because d-tubocurarine, in higher concentrations, disintegrates the actomyosin fibers<sup>18</sup> and also decreases the activity of adenosinetriphosphatase,<sup>19</sup> it is likely that the decreased potassium sensitivity of the muscle in the presence of d-tubocurarine was, at least partly, due to these mechanisms.

Curare, atropine, quinine, strychnine, and veratrine (the latter in high concentrations) were shown to have a curarelike effect, that is, these alkaloids abolish the effect of indirect stimulation on striated muscle in concentrations that do not abolish the effect of direct stimulation.<sup>20, 21</sup> d-Tubocurarine, atropine, amphetamine, codeine, cinchonine, quinine, quinidine, strychnine, and veratrine had a curarelike effect (as shown in the present experiments) in that they decreased the acetylcholine sensitivity of the rectus abdominis muscle in relatively low concentrations but did not decrease the potassium sensitivity in the same concentrations.

#### SUMMARY

1. The effect of eighteen alkaloids on the acetylcholine and potassium sensitivity of striated muscle were investigated.
2. The acetylcholine sensitivity was increased by physostigmine, ephedrine, epinephrine, ergotamine, pilocarpine, morphine, and yohimbine (the latter three in higher concentrations), and was decreased by the other alkaloids tested.
3. The potassium sensitivity was increased in the presence of all alkaloids tested (except epinephrine and cocaine), and was decreased by epinephrine and cocaine, and, in higher concentrations, by d-tubocurarine, strychnine, quinine, quinidine, and atropine.
4. d-Tubocurarine, codeine, atropine, amphetamine, cinchonine, quinidine, quinine, strychnine, and veratrine showed a curarelike effect in that they induced a decrease of acetylcholine sensitivity in low concentrations and such concentrations did not modify the potassium sensitivity of the muscle.

The authors wish to express their gratitude to Smith, Kline, & French Laboratories, Philadelphia, Pa., for the generous supply of amphetamine; to Sandoz Chemical Works, Inc., New York, N. Y., for the ergotamine; to E. R. Squibb & Sons, New York, N. Y., for the d-tubocurarine; and to S. B. Penick and Co., New York, N. Y., for the veratrine.

#### REFERENCES

1. Torda, C., and Wolff, H. G.: Effect of Epinephrine and Physostigmine on the Response of Striated Muscle to Acetylcholine and Potassium, *Am. J. Physiol.* 146: 567, 1946.
2. Gasser, H.: Contractures of Skeletal Muscle, *Physiol. Rev.* 10: 35, 1930.



3. von Muralt, A.: Zusammenhänge zwischen physikalischen und chemischen Vorgängen bei der Muskelkontraktion, *Ergebn. d. Physiol.* 37: 406, 1935.
4. Buchthal, F., and Lindhard, J.: Acetylcholine Block of the Motor End-Plate and Electrical Stimulation of Nerve, *J. Physiol.* 95: 59P, 1939.
5. Henny, G. C., Ashkenaz, E. W., and Spiegel-Adolf, M.: X-ray Diffraction of Muscles. II. *Federation Proc.* 3: 58, 1911.
6. Cicardo, V. H.: Action Comparative du Potassium et de l'acétylcholine sur les Muscles des Batraciens, *Compt. rend. Soc. de Biol.* 132: 505, 1939.
7. Singh, I.: The Effect of the Interaction of Ions, Drugs, and Electrical Stimulation as Indicated by Contraction of the Anterior Retractor of the Byssus of *Mytilus Edulis*, *J. Physiol.* 92: 62, 1938.
- Idem: Properties of Tonic Contractions Produced by Electrical Stimulation of the Anterior Retractor of the Byssus of *Mytilus Edulis*, *J. Physiol.* 94: 1, 1938.
8. Ammon, R.: Die Cholinesterase, *Ergebn. d. Enzymforsch.* 4: 101, 1935.
9. Baeq, Z. M.: Sensibilisation au Potassium par la Vêratriine, *Compt. rend. Soc. de biol.* 130: 1369, 1939.
10. Ammon, R.: Die fermentative Spaltung des Acetylcholins, *Arch. f. d. ges. Physiol.* 233: 486, 1933.
11. Vahlquist, B.: On the Esterase Activity of Human Blood Plasma, *Skandinav. Arch. f. Physiol.* 72: 133, 1935.
12. Bernheim, F., and Bernheim, M. L. C.: Action of Drugs on Cholinesterase of Brain, *J. Pharmacol. & Exper. Therap.* 57: 427, 1936.
13. Harris, M. H., and Harris, R. S.: Effect in Vitro of Curare Alkaloids and Crude Curare Preparations on "True" and Pseudo-Cholinesterase Activity, *Proc. Soc. Exper. Biol. & Med.* 56: 223, 1944.
14. Kahlson, G., and Uvnäs, B.: Die Bedeutung der Acetylcholinesterase sowie der spezifischen Rezeptoren für die Acetylcholinempfindlichkeit kontraktiler Substrate, *Skandinav. Arch. f. Physiol.* 78: 40, 1938.
15. Nachmansohn, D.: Sur l'action de la strychnine, *Compt. rend. Soc. de biol.* 129: 911, 1938.
16. Kaswin, A.: Action inhibitrice des ammoniums quaternaires sur l'activité de la cholinestérase du sérum sanguin, *Compt. rend. Soc. de biol.* 130: 859, 1939.
17. Wright, C. I., and Sabins, J. C.: The Inactivation of Cholinesterase by Morphine, Dilaudid, Codeine, and Desomorphine, *J. Pharmacol. & Exper. Therap.* 78: 375, 1943.
18. McIntyre, A. R., and Braverman, I.: The Actions of Certain Drugs on an Actomyosin-ATP System, *Federation Proc.* 6: 158, 1947.
19. Torda, C., and Wolff, H. G.: Effect of Acetylcholine, Caffeine, and Alkaloids on Activity of Adenosinetriphosphatase. In press.
20. Sollman, T.: A Manual of Pharmacology and its Applications to Therapeutics and Toxicology, Philadelphia, 1942, W. B. Saunders Company.
21. Goodman, L., and Gilman, A.: The Pharmacological Basis of Therapeutics, New York, 1941, The Macmillan Company.

## LABORATORY METHODS

### DILUTION TECHNIQUE FOR RAPID ESTIMATION OF DIHYDROSTREPTOMYCIN AND OTHER ANTIBACTERIAL AGENTS IN BODY FLUIDS

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THE technique to be described is a simple dilution procedure for the rapid estimation of dihydrostreptomycin,<sup>\*,1</sup> streptomycin, penicillin, or other antibacterial agents in a fluid medium. Theoretically, dihydrostreptomycin should be readily assayed by many of the rapid methods used for the determination of streptomycin, but some chemical methods<sup>2-4</sup> are no longer applicable due to modification<sup>1, 5</sup> of the structure. The microbiologic, nephelometric methods<sup>1, 6, 7</sup> are generally impossible to apply to blood, and agar plates<sup>8-10</sup> are laborious and cumbersome when used for infrequent samples. A simple dilution method for rapid estimation of antibiotics in the blood is a clinical and research necessity. Such a technique was not found in the literature.

The following procedure, which has been used successfully for over two years for the determination of streptomycin,<sup>11</sup> is based on the profuse growth of a large inoculum of a sensitive test organism and on its ability to reduce a solution of methylene blue added after growth has begun. The reaction produces a clear, easily read end point. The tubes in which growth has occurred are easily distinguished from those in which it has been inhibited by the antibacterial agent; in the latter, the blue color remains unchanged. While the end point can be determined in three hours and the concentration of the antibiotic can be estimated then, a sharper end point is obtained after four hours.

#### METHOD

The following method is used for the determination of an unknown concentration of dihydrostreptomycin (or streptomycin) in a blood sample, whether whole blood, plasma, or serum. The test organism employed is a strain of *Bacillus prodigiosus* (*Serratia marcescens*) which reduces methylene blue in the presence of 0.3 unit, but not 0.4 unit, of dihydrostreptomycin per cubic centimeter of 1 per cent peptone in physiologic saline solution. Streptomycin in a concentration of 0.4  $\mu$ g also inhibits reduction, while a concentration of 0.3  $\mu$ g per cubic centimeter is not inhibitory.

*Procedure (Blood, Plasma, or Serum).*—To the first tube of a series of ten sterile small test tubes (13 by 100 mm. or 13 by 150 mm.), each containing 1.0 c.c. of physiologic saline

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solution, is added 1.0 c.c. of the blood (citrated or heparinated) to be assayed for dihydrostreptomycin. With a fresh pipette the blood and saline solution are mixed in the first tube and 1.0 c.c. is transferred to the second tube; another fresh pipette is used to mix and transfer 1.0 c.c. to the third tube, and so on; 1.0 c.c. is discarded from the tenth tube, the last of the series.

A control series is set up in an identical manner, using instead of the unknown blood sample defibrinated sheep or rabbit blood or, preferably, horse serum containing 100 units of dihydrostreptomycin per cubic centimeter. To each of the ten tubes in both the unknown and the control series is added 1.0 c.c. of  $10^{-2}$  dilution of a heavy (sixteen to twenty-four hours' growth at  $37^{\circ}\text{C}.$ ) culture of *B. prodigiosus* in physiologic saline solution containing 1.0 per cent peptone. Shake the tubes and incubate at  $37^{\circ}\text{C}.$  for two hours. Then add to each tube 0.2 c.c. of a 1:10,000 solution of methylene blue dissolved in physiologic saline solution. After shaking to obtain an evenly colored mixture, each tube is layered with 0.5 c.c. of mineral oil (liquid petrolatum, U.S.P.). The tubes are then incubated again at  $37^{\circ}\text{C}.$  for another two hours.

To estimate the concentration of dihydrostreptomycin in the unknown blood sample, the number of tubes in the unknown series in which the blue color is unchanged is compared with the number in the control series. Thus, if six tubes in the control series remained blue and the unknown series showed five blue tubes, the latter would contain 50 units; if six tubes in the unknown remained blue, it would contain 100 units, or equal the control; seven blue tubes in the unknown series would indicate a concentration of 200 units, and so forth.

*Procedure (Urine or Aqueous Fecal Extract).*—The same procedure is used for urine and fecal extracts containing antibiotics. Sixteen to twenty-four hour cultures of *Escherichia coli*, Waksman and associates<sup>11, 12</sup> grown at  $37^{\circ}\text{C}.$  in 1 per cent peptone, have been used in  $10^{-3}$  dilutions for the determination of dihydrostreptomycin or streptomycin in such samples. This coliform organism does not produce reduction when dihydrostreptomycin is present in 1.1 units per cubic centimeter or streptomycin in  $1.0\text{ }\mu\text{g}$  per cubic centimeter, but is effective in 1.0 unit of dihydrostreptomycin or  $0.9\text{ }\mu\text{g}$  of streptomycin per cubic centimeter. However, a  $10^{-2}$  dilution of *B. prodigiosus*, which grows more slowly than *Esch. coli*, is preferable.

The high levels of streptomycin present in urinary or fecal samples taken during therapy necessitate a tenfold dilution of the original sample, and the final value for the unknown sample is obtained by multiplying the estimate by ten.

#### DISCUSSION

Sterile glassware is used entirely, but plugs for the test tubes are not necessary. The methylene blue solution and the mineral oil are not sterile. The test may be read one hour after the addition of methylene blue solution and mineral oil, or at any time thereafter. However, reading two to four hours following the second period of incubation is recommended. The number of tubes reduced varies directly with the time of incubation. A late reading not only gives lower sensitivity, but may also render the test inaccurate by allowing contaminants to grow. The latter has not yet been encountered in this laboratory, where no special precautions are observed, and readings at the end of twenty-four hours have been found to be in good agreement with those made earlier, except when the antibiotic was present in low concentration.

In the presence of a 10 per cent or more concentration of blood, for example, in the first and second tubes of the blood dilution series, reduction of methylene blue is not a reliable index of growth manifestations, and non-reduced methylene blue does not necessarily indicate inhibition. The cause of this anomaly is unknown. It is necessary at present to avoid testing whole

blood samples which will give end point results in the initial tubes. Serum and plasma do not react in this way. The testing of serum in place of blood has still other advantages: blood may conveniently be allowed to clot, and serial dilutions may be made without changing pipettes from tube to tube. Under the conditions described, the varying amounts of serum in successive tubes have no noticeable influence on the growth of *B. prodigiosus*.

While sterilization of fresh urine samples is not absolutely necessary, it is recommended that 5 c.c. be heated for twenty to thirty minutes in boiling water before use in antibiotic determination. Dihydrostreptomycin solutions may be sterilized by steam autoclaving for thirty minutes at 115° C. and 15 to 20 pounds of pressure without loss of potency. Solutions of streptomycin, penicillin, or tyrothricin should not be subjected to either treatment. Dilutions of fecal extracts may be tested by the same procedure used for urine.

## APPLICATION

These procedures, which are capable of wide application, can be used with any sensitive organism which grows rapidly under the described conditions. In this laboratory, the aforementioned organisms and *Bacillus subtilis*, *Salmonella typhimurium*, *Staphylococcus aureus* SM, *Staphylococcus aureus* 209P, and *Streptococcus haemolyticus* N.Y.5 have been successfully used in the assay of penicillins, streptothricin, tyrothricin, alkyl quaternary ammonium salts, and some aromatic amidines.

Test data are given in Table I. Values obtained by other methods are included<sup>7, 8, 13-15</sup> for purposes of comparison.

TABLE I

SAMPLE	TEST ORGANISM DILUTION	MEDIUM	TIME (HR.)	ORGANISM INHIBITED (CONC./C.C.)	SAMPLE (CONC./C.C.) (CA.)	KNOWN (CONC./C.C.)	OTHER METHODS (CONC./C.C.)
Dihydrostreptomycin in:							
Horse serum	E.c. 10 <sup>-3</sup>	A	4	1.6 units	200 units	100 units	120 units
Fecal extract	S.t. 10 <sup>-3</sup>	A	4	0.8 unit	1,000 units	1,000 units	----
Blood	B.p. 10 <sup>-2</sup>	A	4	0.8 unit	100 units	100 units	----
Urine	B.p. 10 <sup>-2</sup>	A	4	0.8 unit	1,000 units	1,000 units	1,180 units
Streptomycin in:							
Urine	E.c. 10 <sup>-3</sup>	A	4	0.8 µg	4,000 µg	----	3,800 µg
Serum	B.p. 10 <sup>-2</sup>	A	4	0.2 µg	100 µg	100 µg	90 µg
Fecal extract	B.p. 10 <sup>-2</sup>	A	4	0.4 µg	5,000 µg	----	----
Penicillin in:							
Serum	S.a. 10 <sup>-2</sup>	B	3	0.080 unit	1.25 units	----	1.5 units
Blood	S.a. 10 <sup>-3</sup>	B	6	0.030 unit	12.50 units	25 units	----
Physio. saline	S.a. 10 <sup>-3</sup>	B	6	0.010 unit	50.00 units	50 units	43.5 units
Serum	S.h. 10 <sup>-2</sup>	C	6	0.005 unit	1.25 units	----	1.5 units
Quaternary ammonium salt in:							
Urine	E.c. 10 <sup>-3</sup>	A	4	16.00 µg	2 mg.	2 mg.	2.2 mg.
Water	S.t. 10 <sup>-3</sup>	A	4	0.02 µg	10 mg.	10 mg.	9.8 mg.
Tyrothricin in:							
Water	B.s. 10 <sup>-2</sup>	B	3	1 µg	0.25 mg.	0.25 mg.	----
Streptothricin in:							
Water	P.p. 10 <sup>-2</sup>	B	10	16 units	500 units	----	----

E.c., *Esch. coli*; S.t., *S. typhimurium*; B.p., *prodigiosus*; S.a., *Staph. aureus*; B.S., *B. subtilis*; S.h., *Str. haemolyticus*; P.p., *P. pestis*.

A, 1 per cent peptone; B, hormone broth;<sup>16</sup> C, B with 5 per cent serum.

Mg. (milligram) = 1000 µg (microgram).

## SUMMARY

A simple microbiologic serial dilution technique has been presented in detail for the four-hour estimation of dihydrostreptomycin or streptomycin in whole blood, plasma, serum, urine, or fecal extractions.

The procedure has also been applied to other antibacterial agents, such as penicillins, streptothricin, tyrothricin, quaternary ammonium salts, and aromatic amidines.

## REFERENCES

1. Bartz, Q. R., Controulis, J., Crooks, H. M., Jr., and Rebstock, M. C.: Dihydrostreptomycin, *J. Am. Chem. Soc.* 68: 2163, 1946.
2. Scudi, J. V., Boxer, G. E., and Jelinek, V. C.: A Color Reaction Given by Streptomycin, *Science* 104: 486, 1946.
3. Schenck, J. R., and Spielman, M. A.: Formation of Maltol by the Degradation of Streptomycin, *J. Am. Chem. Soc.* 67: 2276, 1945.
4. Levy, G. B., Schwed, P., and Sackett, J. W.: Polarographic Analysis of Streptomycin, *J. Am. Chem. Soc.* 68: 528, 1946.
5. Peck, R. L., Hoffhine, C. E., Jr., and Folkers, K.: Streptomycins Antibiotics. IX. Dihydrostreptomycin, *J. Am. Chem. Soc.* 68: 1390, 1946.
6. Price, C. W., Nielsen, J. K., and Welch, H.: The Estimation of Streptomycin in Body Fluids, *Science* 103: 56, 1946.
7. Osgood, E. E., and Graham, S. M.: A Simple Rapid Method for Assay of Bactericidal and Bacteriostatic Agents, *Am. J. Clin. Path.* 17: 93, 1947.
8. Stebbins, R. B., and Robinson, H. J.: A Method for the Determination of Streptomycin in Body Fluids, *Proc. Soc. Exper. Biol. & Med.* 59: 255, 1945.
9. Loo, Y. H., Skell, P. S., Thornberg, H. H., Ehrlich, J., McGuire, J. M., Savage, G. M., and Sylvester, J. C.: Assay of Streptomycin by the Paper Disc Plate Method, *J. Bact.* 50: 701, 1945.
10. Forgacs, J., Kornegay, G. B., and Henley, T. F.: Studies on Streptomycin. I. Assay in Body Fluids, *J. LAB. & CLIN. MED.* 31: 514, 1946.
11. Schatz, A., Bugie, E., and Waksman, S. A.: Streptomycin, a Substance Exhibiting Antibiotic Activity Against Gram-Positive and Gram-Negative Bacteria, *Proc. Soc. Exper. Biol. & Med.* 55: 66, 1944.
12. Waksman, S. A., and Schatz, A.: Streptomycin, *J. Am. Pharm. A.* 34: 273, 1945.
13. Goyan, F. M., Dufrenoy, J., Strait, L. A., and Pratt, R.: A Three-Hour "Physical Development" Cup-Plate Assay for Penicillin, *J. Am. Pharm. A. (Scient. Ed.)* 36: 65, 1947.
14. Fleming, A.: In Vitro Tests of Penicillin Potency, *Lancet* 1: 732, 1942.
15. Auerbach, M. E.: Colorimetric Assay of Quaternary Ammonium Salts, *Indust. & Engin. Chem. (Anal. Ed.)* 16: 739, 1944.
16. Meyer, K. F., and Batchelder, A. P.: Selective Mediums in Diagnosis of Rodent Plague, *J. Infect. Dis.* 39: 370, 1926.
17. Huntoon, F. M.: "Hormone" Medium, *J. Infect. Dis.* 23: 169, 1918.

# ESTIMATION OF URIC ACID IN SERUM AND WHOLE BLOOD BY AN ELECTROPHOTOMETRIC MODIFICATION OF FOLIN'S METHOD

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THE purpose of this communication is to report a study of the estimation of uric acid in serum and in whole blood by an electrophotometric modification of Folin's direct macromethod.<sup>1,2</sup> Details of the adaptation of Folin's technique to the electrophotometer, recoveries of uric acid from serum and whole blood, and estimations of the uric acid content of serum and whole blood of normal subjects are presented.

This investigation was stimulated by a report by Jacobson<sup>3</sup> of the satisfactory results of the application to serum of Folin's direct method of estimation of blood uric acid.<sup>1</sup> In thirty experiments, in which uric acid was added to serum (or plasma), the average recovery was 90 per cent and individual recoveries were 80 per cent or higher in all but one instance. The upper limit of the normal range of serum uric acid concentration was 6.0 mg. per cent, and values above this were found in 174 of 177 tests on twenty-one patients with gout. These recoveries and the demonstration of this high incidence of hyperuricemia in gout are in contrast with the findings of many investigators. Thus, in 1941, Bulger and Johns<sup>4</sup> stated that the uncertainty regarding the reliability of methods of estimation of blood uric acid was so great that some laboratories no longer attempted these tests. We were therefore prompted to study Folin's method with special reference to its application to serum and, since whole blood is commonly used for estimation of uric acid, to compare results obtained with serum and whole blood.<sup>5</sup>

*Electrophotometric Modification of Folin's Direct Macromethod.*—Preliminary experiments with Folin's technique showed that the color of the blank was not constant. At times it was pale to moderately deep yellow; at other times, although pale yellow at first, it rapidly became pale green. These variations appeared to be due to impurities in the sodium cyanide used in the urea-cyanide reagent† and, to a less extent, to very small changes in the proportions of sodium tungstate and phosphoric acid in the uric acid reagent.<sup>3</sup> A change in the blank occurred whenever a new lot of sodium cyanide was used and sometimes when a new uric acid reagent was prepared. It was therefore decided to adapt the technique to the electrophotometer, since this would permit a correction for the blank with each estimation.

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\*In all tests with whole blood the anticoagulant was sodium oxalate and red cells were laked before precipitation of proteins.<sup>6</sup> Unlaked blood filtrates<sup>7</sup> were not used since they yield values which are influenced by a variable degree of extraction of chromogenic material from the red cells.<sup>4</sup>

†Folin<sup>2</sup> recommended the use of Merck's reagent sodium cyanide. In our experience this does not ensure constancy of the blank.

A Fisher electrophotometer with two photoelectric cells, null adjustment, microabsorption cells and a filter of maximum transmission at 525  $m\mu$  was used.

Experiments with aqueous solutions of uric acid showed concentration to be directly proportional to the negative logarithm of the transmission factor with amounts of uric acid below 0.6 mg. per cent only (equivalent to a serum or blood uric acid of 6 mg. per cent). The rate of color development was variable and appeared to be markedly influenced by impurities in the sodium cyanide used in the urea-cyanide reagent. With some lots of cyanide color development was slow and a deep blue color was not formed until thirty to forty minutes after addition of uric acid reagent. With other lots color development was more rapid, the blue color was deep at the end of twenty minutes and was replaced by a green color between thirty and forty minutes. A calibration curve could not be used and a standard, equivalent to a blood uric acid of 4 mg. per cent, was set up with each unknown as described by Folin.<sup>1, 3</sup>

Experiments with Folin-Wu filtrates representing 1 in 10 dilutions of serum and blood<sup>3, 6</sup> showed their behavior to be similar to that of aqueous solutions. Rate of color development was variable but paralleled that of the standard set up at the same time with the same reagents.

On the basis of these findings, Folin's direct macromethod was adapted to the electrophotometer as follows. A standard, equivalent to a blood uric acid of 4 mg. per cent, and a blank were set up with each unknown. Readings were made at any time between twenty and forty minutes after addition of uric acid reagent. With the blank in position in the electrophotometer, the galvanometer was adjusted to read zero on the logarithmic scale. Readings of the standard and the unknown were then made. Since the colors of the blank, the standard, and the unknown were not stable, readings were made in as rapid succession as possible. The concentration of uric acid in the unknown was calculated from the readings of the logarithmic scale of the galvanometer. If the value obtained was higher than 6 mg. per cent, the test was repeated with a filtrate diluted with water to obtain a final reading in the range of direct proportionality between concentration and logarithmic scale readings.

The modifications introduced in adapting the technique to the electrophotometer only have been described; otherwise the preparation of reagents and procedure of the test were as described by Folin.<sup>1-3</sup>

*Recovery of Uric Acid From Serum and Whole Blood.*—Uric acid was added to serum and whole blood in concentrations ranging between 2 and 10 mg. per cent. Filtrates, representing dilutions of serum or blood of 1 in 10, were prepared by the Folin-Wu procedure<sup>3, 6</sup> and estimations before and after addition of uric acid were made by the electrophotometric modification of Folin's direct macromethod. The results (Table I) show that recoveries from whole blood were lower and more variable than those from serum. Demonstration of the inaccuracies involved in analysis of the uric acid content of whole blood is not new and the factors responsible for poor recoveries have been discussed

in the literature.<sup>1, 2, 3</sup> The figures reported here are of interest in providing a comparison between recoveries from serum and whole blood by the same technique.

TABLE I. RECOVERIES OF URIC ACID FROM SERUM AND WHOLE BLOOD

	TOTAL NUMBER OF EXPERIMENTS	RECOVERY OF ADDED URIC ACID <sup>a</sup>			NUMBER OF EXPERIMENTS WITH RECOVERIES OF 85% OR MORE
		MAXIMUM	MINIMUM	AVERAGE	
Serum	30	110%	78%	94%	29
Whole blood	30	105%	55%	83%	15

<sup>a</sup>Recoveries were calculated by the formula  $\frac{U_2 - U_1}{a} \times 100\%$ , in which  $U_1$  and  $U_2$  were amounts of uric acid found before and after addition of uric acid, respectively, and  $a$  was the amount added. In some publications recovery is calculated as  $\frac{U_2}{U_1 + a} \times 100\%$ . The latter method yields higher figures, the discrepancy is especially marked when  $U_1$  is high and  $a$  is low.

Variations in the color of the blank and in the rates of color development by standards and unknowns had no influence on recoveries. It should be noted here that these recovery experiments were not done with only one set of reagents. Six lots of sodium cyanide, representing products of three manufacturers, and four preparations of uric acid reagent were used.

These results indicate that serum is to be preferred to whole blood for estimation of uric acid by Folin's method.

*Normal Values of Uric Acid Content of Serum and Whole Blood.*—Blood was collected from 100 normal individuals with no family history of gout. There were 63 men and 37 women. The maximum, minimum, and average ages were 58, 15, and 29 years respectively. Samples were obtained in the morning one to three hours after breakfast and were not collected under oil.<sup>4</sup> The latter precaution was omitted since it is inconvenient in routine work and we wished to determine normal values with samples collected under the usual conditions of exposure to air. Uric acid in Folin-Wu filtrates of serum and whole blood was estimated by the electrophotometric modification of Folin's direct macro-method.

The results are shown in Tables II and III. As expected from the recoveries, average values obtained with whole blood were lower than those obtained with serum. Individual values showed considerable variation. Thus, whole blood values were equal to or higher than serum values in 15 of the 100 tests

<sup>a</sup>The ratios of the difference between the means to the probable error of the difference show the differences between whole blood and serum values to be statistically significant. Statistical formulas used

$$\sigma \text{ sample} = \sqrt{\frac{\sum v^2}{n}}$$

$$P. E. \text{ sample} = 0.6745 \times \sigma \text{ sample}$$

$$\sigma \text{ mean} = \frac{\sigma \text{ sample}}{\sqrt{n}}$$

$$P. E. \text{ mean} = 0.6745 \times \sigma \text{ mean}$$

$$\sigma(M_1 - M_2) = \sqrt{(\sigma M_1)^2 + (\sigma M_2)^2}$$

$$P. E. (M_1 - M_2) = 0.6745 \times \sigma(M_1 - M_2)$$

In these formulas,  $\sigma$ , standard deviation,  $v$ , individual deviation from mean,  $n$ , number of measurements,  $P. E.$ , probable error,  $M_1$  and  $M_2$ , means of two series of measurements.



and, among the remaining 85, differences between serum and whole blood values varied between 0.1 and 2.9 mg. per cent. In individual cases, the uric acid content of serum cannot be predicted from the value obtained using whole blood.

TABLE II. SERUM URIC ACID IN ONE HUNDRED NORMAL SUBJECTS

	NUMBER OF TESTS	SERUM URIC ACID (MG. PER CENT)				
		MAXIMUM	MINIMUM	AVERAGE	PROBABLE ERROR SAMPLE	PROBABLE ERROR MEAN
All cases	100	6.9	2.9	4.5	$\pm 0.6$	$\pm 0.06$
Men	63	6.9	2.9	4.8	$\pm 0.6$	$\pm 0.08$
Women	37	5.7	2.9	4.0	$\pm 0.4$	$\pm 0.07$

Our normal values of serum uric acid are slightly higher than those reported by Jacobson.<sup>4</sup> This is probably explained by the fact that our samples of blood were not protected from exposure to air, whereas Jacobson collected blood under oil. This investigator has shown that exposure of blood to air may increase the apparent uric acid content of serum by as much as 0.6 mg. per cent. It should also be noted that Jacobson's tests were done while the subject was in the fasting state, whereas those reported here were done one to three hours after breakfast. However, there was no evidence that our highest values were related to consumption of breakfasts of high purine contents.

TABLE III. WHOLE BLOOD URIC ACID IN ONE HUNDRED NORMAL SUBJECTS

	NUMBER OF TESTS	WHOLE BLOOD URIC ACID (MG. PER CENT)				
		MAXIMUM	MINIMUM	AVERAGE	PROBABLE ERROR SAMPLE	PROBABLE ERROR MEAN
All cases	100	6.4	2.2	3.8	$\pm 0.6$	$\pm 0.06$
Men	63	6.4	2.5	4.1	$\pm 0.6$	$\pm 0.08$
Women	37	4.4	2.2	3.2	$\pm 0.4$	$\pm 0.07$

Nothing would be gained from a detailed comparison of our data with those obtained by other techniques<sup>5, 9-13</sup> since values depend to a considerable extent on the technique employed. In general, values obtained with serum or plasma are higher than those obtained with whole blood and indicate the upper limit of the normal range for serum or plasma to be 6 to 7 mg. per cent.<sup>14</sup>

As shown in Tables II and III our data agree with those reported elsewhere<sup>5, 10, 12</sup> in demonstrating lower values in women than in men.<sup>2</sup>

## SUMMARY

1. An electrophotometric modification of Folin's direct macromethod of estimation of uric acid in blood is described. This was developed to eliminate errors due to variable blanks met with in this technique.

2. With this method recoveries of uric acid from serum were higher and less variable than those from whole blood. Serum is therefore to be preferred to whole blood for estimation of uric acid by this technique.

\*The ratios of the difference between the means to the probable error of the difference show these differences between men and women to be statistically significant.

3. Estimations of the uric acid content of whole blood and serum were made in 100 normal individuals with no family history of gout. The average values obtained with whole blood were significantly lower than those obtained with serum, but individual values showed considerable variation. In individual cases the uric acid content of serum cannot be predicted from analysis of whole blood.

The normal range of concentration of uric acid in serum was 2.9 to 6.9 mg. per cent.

Whole blood and serum uric acid concentrations were significantly lower in women than in men.

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#### REFERENCES

1. Folin, O.: Standardized Methods for the Determination of Uric Acid in Unlaked Blood and in Urine, *J. Biol. Chem.* 101: 111, 1933.
2. Folin, O.: The Preparation of Sodium Tungstate Free From Molybdate, Together With a Simplified Process for the Preparation of a Correct Uric Acid Reagent (and Some Comments), *J. Biol. Chem.* 106: 311, 1934.
3. Folin, O.: *Laboratory Manual of Biological Chemistry*, ed. 5, New York, 1934, D. Appleton-Century Company, Inc.
4. Jacobson, B. M.: The Uric Acid in the Serum of Gouty and of Non-gouty Individuals: Its Determination by Folin's Recent Method and Its Significance in the Diagnosis of Gout, *Ann. Int. Med.* 11: 1277, 1938.
5. Bulger, H. A., and Johns, H. E.: The Determination of Plasma Uric Acid, *J. Biol. Chem.* 140: 427, 1941.
6. Folin, O., and Wu, H.: A System of Blood Analysis, *J. Biol. Chem.* 38: 81, 1919.
7. Folin, O.: Unlaked Blood as a Basis for Blood Analysis, *J. Biol. Chem.* 86: 173, 1930.
8. Folin, O.: A System of Blood Analysis. Supplement IV. A Revision of the Method for Determining Uric Acid, *J. Biol. Chem.* 54: 153, 1922.
9. Blauch, M. B., and Koch, F. C.: A New Method for the Determination of Uric Acid in Blood, With Uricase, *J. Biol. Chem.* 130: 443, 1939.
10. Brøchner-Mortensen, K.: The Uric Acid Content in Blood and Urine in Health and Disease, *Medicine* 19: 161, 1940.
11. Mull, J. W.: Determination of Uric Acid in Whole Blood and Serum, *J. Lab. & Clin. Med.* 28: 1038, 1943.
12. Brown, H.: The Determination of Uric Acid in Human Blood, *J. Biol. Chem.* 158: 601, 1945.
13. Block, W. D., and Geib, N. C.: An Enzymatic Method for the Determination of Uric Acid in Whole Blood, *J. Biol. Chem.* 168: 747, 1947.
14. Peters, J. P., and Van Slyke, D. D.: *Quantitative Clinical Chemistry*. Interpretations, vol. 1, ed. 2, Baltimore, 1946, Williams & Wilkins Company, pp. 957-958.

## A DERMAL PLETHYSMOGRAPH FOR THE RECORDING OF SKIN-WHEALING REACTIONS

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PREVIOUS comparative studies on intradermal wheals, initiated shortly after the introduction of the McClure-Aldrich saline wheal disappearance tests,<sup>1, 2</sup> were disappointing because of lack of quantitative methods for the measurement of wheal volumes. The first effort at quantitation was probably that of Schade<sup>3</sup> in 1912 who used a weighted disk placed on top of saline wheals and recorded kymographically the slow sinking and disappearance of these wheals. Alexander and co-workers<sup>4-6</sup> traced the irregular wheal boundary with a planimeter and used the recording as an index of wheal size. Gowen<sup>7</sup> suggested a practical clinical method of approximating the size of wheals by the use of a thin 4 cm. square glass with three circles, 1 cm., 2 cm., and 3 cm. in diameter, marked on its surface. For measurements, the glass square was placed over the wheal with the point of injection in the center of the 1 cm. circle. A rubber stamp with circles of the same size was used to record and transfer a rough outline of the wheal size to the patient's chart.

Intradermal wheals have been used in animals and man to compare the degree of irritation caused by local anesthetics.<sup>8</sup> An accurate means of wheal measurement would suggest extending this simple test to study the intradermal tissue tolerance of other new drugs. The tendency of many drugs to cause an inflammatory reaction or cellular irritation, as well as the characteristic curve of absorption, might thus be determined by the use of various concentrations of the test substance. The results of intradermal injections could then be correlated with the intramuscular and subcutaneous reactions to the drug. During the course of studies on new local anesthetics a distinct need was felt for a more accurate means of measuring wheals and cutaneous reaction to intradermal injections. Accordingly the following dermal plethysmograph was designed.

### APPARATUS

The dermal plethysmograph (Fig. 1) consists of a 20 to 30 cm. glass capillary tube (bore, approximately 1 mm.) opening into a hollowed-out rubber base. Using rubber cement, a thin rubber membrane is cemented to the rubber base of the plethysmograph and the edges of the membrane are then trimmed with scissors. When the tube and rubber base are filled with an 80 per cent alcoholic solution of toluidine or methylene blue, any pressure against the rubber membrane causes the blue solution to rise in the tube.

From the Department of Pharmacology, University of Illinois College of Medicine.  
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\*Submitted in partial fulfillment of the requirements for the degree of Master of Science in Pharmacology.

When weighted with sufficient lead to make the entire instrument weigh 100 grams it is extremely sensitive to volumetric changes of the skin surface. The pulse is seen as a regular rise and fall of the blue column as the instrument rests on the subject's arm. Greater weight may cause a gradual depression of the supporting skin and a resultant rise of the blue column of fluid over a forty-five minute period. With an external diameter of 37 mm. and an internal opening of 21 mm., the instrument applies 100 grams of weight over

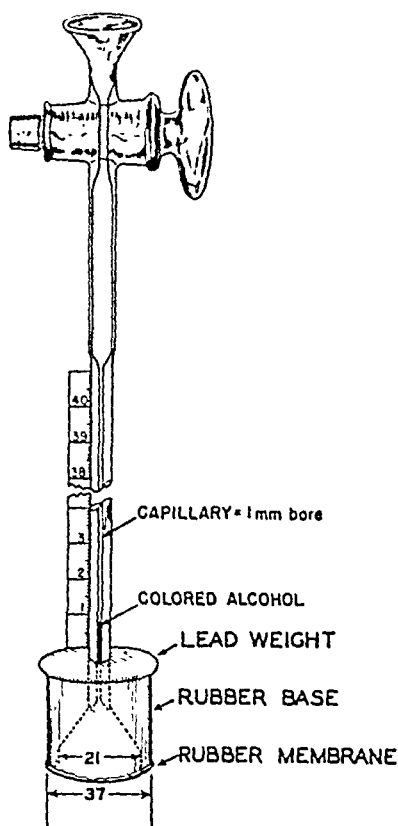


Fig. 1.—Dermal plethysmograph.

an area of 728 sq. mm., or approximately 50 grams per 350 square millimeters. In this regard Schade<sup>3</sup> found that 50 grams per 50 sq. mm. did not exceed the elastic limits of the skin when applied for a two-minute period. Control readings indicate that 100 grams per 728 sq. mm. does not exceed the elastic limits when applied for a forty-five minute period, since no spontaneous rise occurs during this time. That changes in skin temperature are not important in the readings of the skin plethysmograph is shown by Fig. 2. The bases of three plethysmographs were immersed to a depth of 2 mm. in a water bath and the temperature was raised slowly from 22 (room temperature) to 37.5° C. Over the temperature range encountered in the skin of the forearm (33 to 37° C.) the readings of the plethysmographs increased only 1 to 2 mm., which

is not sufficient to necessitate a correction. When the capillary is calibrated with mercury it is found that a capillary of approximately 1.0 mm. bore will contain 0.009 c.cm. of water per centimeter length. Thus with individually calibrated capillaries, graph paper may be used to measure the changes in volume of the skin surface. Knowing that the wheal is made in the dermis,<sup>9, 10</sup>

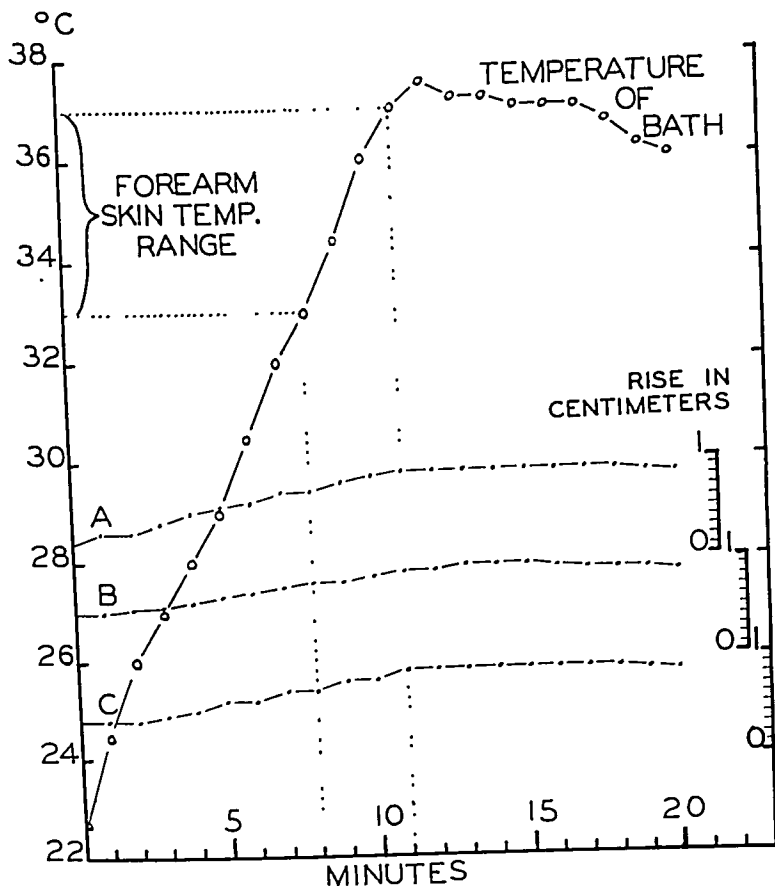


Fig 2.—The possible effect of skin temperature on the volumetric readings of the dermal plethysmograph. A, B, C, readings in centimeters of three dermal plethysmographs.

one can assume that at least one-half is submerged in the soft subcutaneous tissues so that only the top half of any given wheal could possibly be measured. The instrument is calculated on this basis to be 63 per cent effective. That is, when 0.2 c.c. wheals were made with serum, the total volume measured is 0.063 c.c. rather than the theoretic value of 0.10 cubic centimeter.

#### PROCEDURE

Most consistent results are obtained with a trained and relaxed subject reclining on a bed with the arm immobilized with small sand bags. A series of control readings are taken on a level area of the flexor surface of the subject's forearm by lifting and replacing the plethysmograph several times. The

average of these control readings is taken as a base reading. Each time before removing the plethysmograph from the skin the stopcock should be closed to prevent bulging of the rubber membrane due to pressure from the column of fluid. It is opened upon replacing. After the control readings have reached a constant value, the instrument is removed from the arm and a wheal is raised in the exact center of the area where the dermal plethysmograph had been resting. The wheal is made by injecting intradermally 0.2 c.c. of the sterile test solution using a  $\frac{1}{4}$  c.c. tuberculin syringe and a 27-gauge one-fourth inch needle. The stop watch is started after the needle is inserted and immediately before the solution is injected. The dermal plethysmograph is replaced over the identical area, and readings are taken at thirty and sixty seconds and at every minute thereafter for a period of forty-five minutes.

With the enormous pressures required for whealing,<sup>11</sup> some variation in wheal size may result from leakage of a test solution either back around the syringe plunger in a poorly fitting syringe or between the syringe and the needle. To avoid this in quantitative work, each syringe must be carefully tested for leaks by the following procedure: a needle is firmly attached to the syringe, then inserted into a solid rubber stopper; if pressure exerted on the syringe causes the fluid in the syringe to escape at the bevel or back along the piston, the syringe is discarded. Puncturing of the basal layer of the corium (as by poor technique) also causes a smaller and more rapidly disappearing wheal. (Some evidence has been obtained which indicates that a markedly irritating solution may regularly result in smaller wheals.) Finally, the primary factors in the size and disappearance of wheals are the varying abilities of the solutions to spread between the cell walls, to penetrate the tissues and the cell walls, to cause cellular swelling, to increase capillary permeability, and to cause delayed inflammatory reactions.

Since the human subject must lie supine for forty-five minutes we have found it expedient to use three dermal plethysmographs simultaneously and make three wheals in rapid succession in the skin of the subject's forearm. The tabulation of data from three plethysmographs at minute intervals is easily within the skill of a trained operator.

#### RESULTS

*Example I. Reaction to Insect Toxin.*—Subject W. F., a 21-year-old man known to have an immediate whealing reaction to mosquito bites, was chosen for this experiment. A single mosquito (*Aedes aegypti*) was allowed to bite the subject in the volar surface of the forearm. After the mosquito had become engorged and had withdrawn his proboscis, the dermal plethysmograph was placed on the site and development of the wheal measured (Fig. 3, A). The experiment, when repeated the next day, produced a similar type of whealing reaction (Fig. 3, B). The response curves are sufficiently analogous to indicate the usefulness of this method of recording when immediate reactions to an injected substance are anticipated.

*Example II. Reaction to Histamine Diphosphate.*—Two subjects were chosen for this experiment. Subject A, a 20-year-old man, was known to

react quite markedly to intradermal histamine, while Subject B, a 38-year-old man, was known to have a less marked response. Neither subject had an allergic history. The skin of the subject was cleaned with 70 per cent alcohol, and a drop of histamine diphosphate, 1:1,000, was placed on the clean dry skin. A sterile 27-gauge one-fourth inch needle was then used to make two punctures through the dermis beneath the drop of histamine. The excess histamine was blotted off with gauze and the recording instrument was placed over the site. This technique results in a uniform whealing response when used in

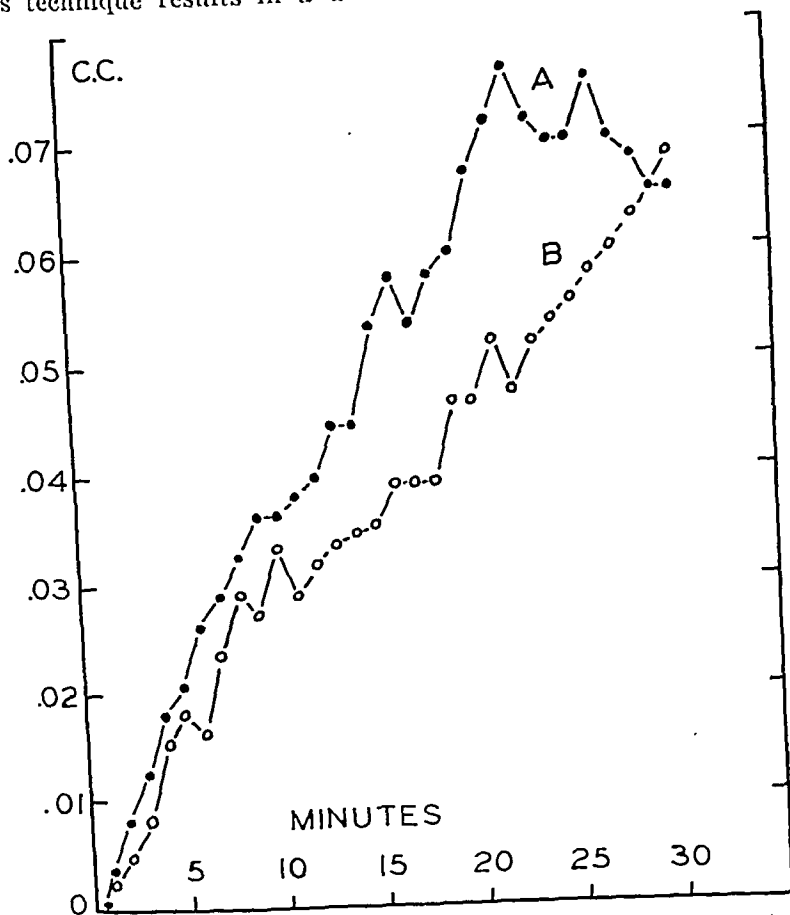


Fig. 3.—The response of a normal subject to mosquito bites (*Aedes Egypti*) on two successive days.

experimental subjects during the summer months. (In winter months we have found the skin to be much less responsive.) It is evident from inspection of the curves obtained on three successive days (Fig. 4) in each subject that Subject A has a consistently greater response to the whealing action of histamine than Subject B who has a consistently minimal response. These separate families of curves are distinct and probably reflect the sensitivity of these two subjects when histamine is applied in this manner.

*Example III. Disappearance Curves of Saline and Glucose.*—Previous examples of the application of the dermal plethysmograph have shown charac-

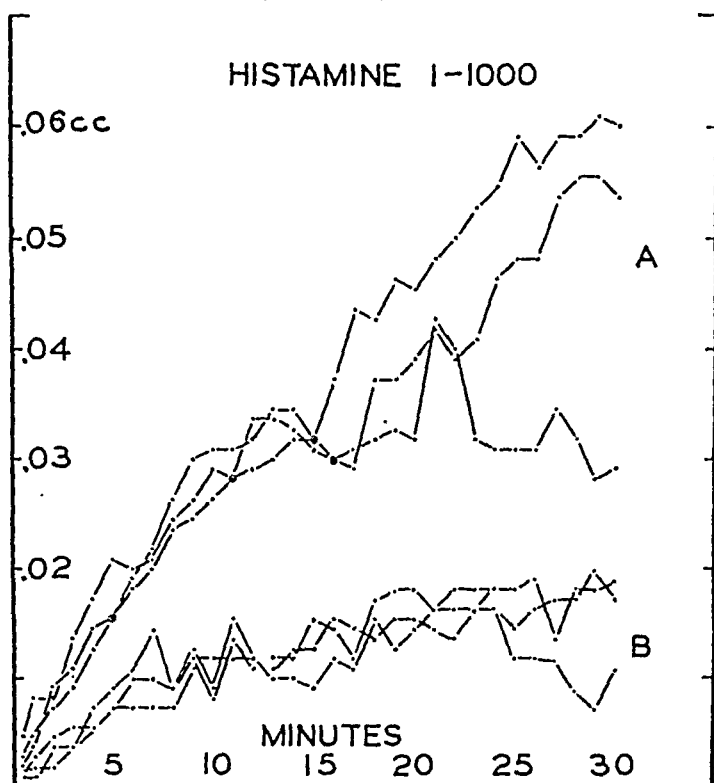


Fig. 4.—The response of two normal subjects, A and B, to histamine on three successive days.

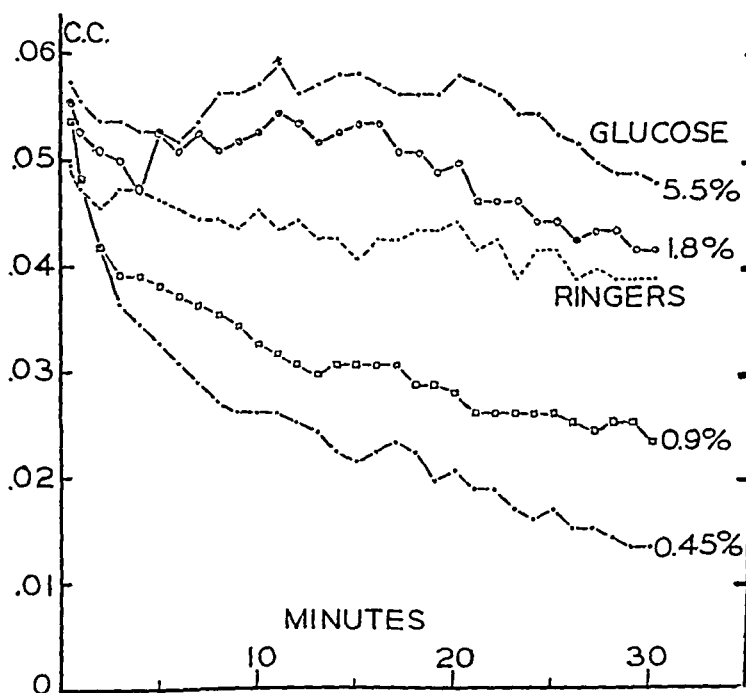


Fig. 5.—Wheal disappearance times (average of five subjects) when solutions of varying tonicity are injected intradermally.



teristic curves of wheal growth. Wheals of various substances in solutions (such as local anesthetics, irritant drugs, and saline) can be made, the wheal size measured, and a characteristic wheal disappearance curve obtained for each substance. The average disappearance curve of five subjects injected with various saline solutions and glucose is characteristic and predictable (Fig. 5). Clinically we know that 5.5 per cent glucose solution is more irritating than 0.9 per cent NaCl solution. The rising plateau curve obtained with glucose simulates the curves of other irritant substances such as nupercaine 1:500 or diothane 1:500.\* Sodium chloride, 0.45 per cent, most closely approximates the saline level of the extracellular fluid and in these experiments disappears most rapidly from an intradermal site. Sodium chloride, 1.8 per cent, has an irritant quality similar to that of glucose.

## SUMMARY

A dermal plethysmograph (whealometer) has been constructed which measures with a known degree of accuracy the growth and disappearance rate of dermal wheals. Wheal measurement is suggested as a useful procedure for the study of histamine response or other immediate whealing responses and for the determination of the rate of disappearance of various substances injected intradermally.

## REFERENCES

1. McClure, W. B., and C. A. Aldrich: Time Required for Disappearance of Intradermally Injected Salt Solution, *J. A. M. A.* 81: 293-294, 1923.
2. Aldrich, C. A., and W. B. McClure: Intradermal Salt Solution Test; Its Prognostic Value in Nephritis With Generalized Edema, *J. A. M. A.* 82: 1425-1428, 1924.
3. Schade, H.: Untersuchungen zur Organfunktion des Bindegewebes. Die Elastizitätsfunktion des Bindegewebes und die intervital Messung ihrer Störungen, *Ztschr. f. exper. Path. u. Therap.* Berl. 11: 309-399, 1912.
4. Alexander, H. L., Harter, J. O., and McConnell, F. S.: Observations on the Formations of Wheals Induced by Allergens and by Histamine, *Proc. Soc. Exper. Biol. & Med.* 27: 484-486, 1930.
5. Weaver, C. W. K., McConnell, P. H., and Alexander, H. L.: Influence of Calcium Concentration on Histamine Wheals, *J. Clin. Investigation* 11: 195-203, 1932.
6. McConnell, F. S., Weaver, W. K., and Alexander, H. L.: Effects of Variation of CO<sub>2</sub> Combining Power of Blood on Histamine Wheals, *J. Clin. Investigation* 11: 205-209, 1932.
7. Gowen, G. H.: A Practical Method of Measuring and Recording Skin Test Reaction, *J. Lab. & Clin. Med.* 22: 187-191, 1936.
8. Meeker, W. R.: Studies in Local Anesthesia. Local Tissue Reactions, *J. Lab. & Clin. Med.* 11: 474-478, 1926.
9. White, H. L., and Irvine-Jones, E.: Factors Affecting the Disappearance Time of Intradermal Injection, *Am. Heart J.* 2: 517, 1927.
10. Taylor, F. W.: The Actual Site in the Skin of Intradermal Injection, *J. A. M. A.* 111: 2475-76, 1938.
11. Moss, W. G., and Pfeiffer, C. C.: Pressures Required to Produce Intradermal Wheals in Normal Human Subjects, *Proc. Soc. Exper. Biol. & Med.* 63: 44-46, 1946.

\*To be reported in a paper on local anesthetics.

# PROCEEDINGS OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

Twentieth Annual Meeting  
Chicago, Ill., Oct. 31 and Nov. 1, 1947

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## PROGRAM

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SCIENTIFIC PROGRAM—OCTOBER 31, 1947

FRIDAY MORNING, 9:15 A.M.

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### 1. EFFECT OF URETHANE ON LEUCEMIA

PAUL L. BEDINGER, M.D. (BY INVITATION), HENRY G. PONCHER, M.D., AND  
LOUIS R. LIMARZI, M.D., CHICAGO, ILL.

Recent investigation has shown that urethane (ethyl carbamate) produces a marked drop in the leucocyte count of both human leucemia and mouse myelogenous leucemia. Normal leucocytes appear to be more refractory to urethane than do leucemic cells. In human leucemia following the oral administration of urethane over a period of several months a favorable effect has been reported in the subjective symptoms, lymphadenopathy, hepatosplenomegaly, and the leucocyte blood pattern. The exact action of urethane is not understood, but a mechanism of inhibition of mitosis of immature cells has been suggested.

Seventeen children and adults with acute leucemia, fourteen patients with chronic lymphatic leucemia, four with chronic myeloid leucemia, and one with monocytic leucemia (Schilling type) were treated with urethane. The drug was given orally in simple syrup or as  $7\frac{1}{2}$  grain tablets. Because of intolerance in some cases, mainly shown by nausea and vomiting, 5 grain enteric-coated tablets proved satisfactory. The average daily dose of the drug ranged from 1 to 5 Gm. given over a period of two days to six months.

In the patients with acute leucemia little or no benefit was observed from the use of urethane, although a rapid and marked fall in the leucocyte count was noted in all cases. With the exception of one child who showed an apparent temporary remission in the blood and bone marrow pattern without clinical improvement, all the other patients still presented the blood and bone marrow findings of acute leucemia. The severe anemia and thrombopenia necessitated the use of frequent blood transfusions in spite of the drop in leucocyte count during the administration of urethane. Terminal cases of acute leucemia showed a rise in the leucocyte count in spite of the administration of urethane. Eleven of these patients have died and in several of the cases at autopsy the hemopoietic organs, as well as the others, showed a general infiltration of tissues by leucemic cells.

In chronic myeloid leucemia and chronic lymphatic leucemia urethane produces a gradual or rapid fall in the leucocyte count with apparent symptomatic improvement. Enlarged lymph nodes and enlarged spleen decreased in size, but in no case did they disappear. Although a more mature type of blood pattern was observed in the patients with chronic myeloid leucemia following treatment, immature cells of the myeloid series were always present in the

peripheral blood. In the patients with chronic lymphatic leucemia, the fall in the leucocyte count was associated with an improvement in the blood and bone marrow picture, although in no case was a complete hematologic remission induced by the drug. Improvement in the hemoglobin and erythrocyte count was noted in several cases. There is some indication that remissions induced by roentgen treatment may be prolonged by the use of urethane. No clinical or hematologic improvement was noted in the patient with monocytic leucemia.

Urethane produces varying degrees of toxic granulations in the neutrophilic leucocytes which is especially marked in the cases of acute leucemia. Urethane was without effect on the basal metabolic rate and certain blood chemical constituents including uric acid. The tentative conclusions are that (1) in patients with acute leucemia urethane in oral doses of 1 to 5 Gm. daily will produce moderate to marked fall in the leucocyte count during a period of one to several weeks; (2) in patients with chronic lymphatic leucemia and chronic myeloid leucemia there is a tendency for the blood pattern to approach a more mature type with a decrease in the size of the lymph nodes and spleen; (3) the temporary remission induced by the drug in chronic leucemia is never as complete as that following roentgen therapy; (4) there is no indication that urethane has any permanent effect on the leucemic process.

## 2. VARIATIONS IN BLOOD PLATELETS IN ALLERGIC INDIVIDUALS FOLLOWING THE INGESTION OF TEST FOODS

FREDERICK J. POHLE, M.D., AND EPHRAIM B. COHEN, M.D. (BY INVITATION)  
MADISON, WIS.

Purpura as a manifestation of allergy is well known. Such hemorrhagic disorders are usually nonthrombocytopenic, although in rare instances reference has been made to cases of thrombocytopenic purpura presumably due to a food sensitivity. Squier and Madison, particularly, have emphasized the role of food allergy in the etiology of certain cases of thrombocytopenic purpura. The purpose of the present investigation was to quantitate the platelet response in allergic individuals to the ingestion of test foods.

Twenty sets of observations were made on sixteen allergic subjects. Under basal conditions, leucocyte and platelet counts were performed twice at fifteen-minute intervals prior to ingestion of a standard portion of a test food to which the subject was known to be sensitive. The postprandial blood counts were done at fifteen, thirty, sixty, and ninety minutes. Tourniquet tests were done immediately prior to the feeding and were repeated on the opposite arm sixty minutes later. Platelets were enumerated by one of us (F. J. P.) employing a direct method which, in our hands, admits a technical error of approximately  $\pm 4$  per cent.

A positive leucopenic index, as evidenced by a postprandial decrease of 10 per cent or more as suggested by Randolph, was demonstrated in seventeen of the twenty studies. On the same basis, a positive "thrombopenic index" was observed fourteen times. The average change in the leucocyte count for the entire group was -22 per cent; the average change in the platelet count was -19 per cent. In fifteen of the twenty sets of observations the subject demonstrated clinical manifestations of sensitivity to the test food. The tourniquet test became positive after the ingestion of the offending food in two individuals. These two patients showed a decrease in platelets of 31 to 33 per cent, respectively. Similar observations of the leucocyte and platelet counts and the tourniquet test were made on a control group of nonallergic individuals, none of whom demonstrated these changes.

These data suggest that this clinical method may aid in the detection of food allergies and that this type of observation may be of value in the study and management of certain thrombopenic states.

### 3. NEUTRALIZATION OF HEPARIN WITH PROTAMINE (SALMINE)

THOMAS W. PARKIN, M.D. (BY INVITATION), AND WALTER F. KVALE, M.D.  
ROCHESTER, MINN.

Hemorrhage due to a prolonged blood coagulation time resulting from the administration of heparin can be controlled only by discontinuing the administration of heparin and by giving blood transfusion. The effects of neither of these procedures may be sufficiently prompt to prevent the hemorrhage from reaching serious proportions.

Certain protamines are known to neutralize heparin *in vitro* and in animals. Protamines also appear to have certain toxic effects when given to different species of animals. The assumption appears in the literature that on the basis of animal studies protamines cannot be administered safely to human beings. On the contrary, in 1938, Jorpes reported from Sweden that the protamine, clupeine, can be injected intravenously in man to neutralize heparin. In view of the conflicting reports in the literature, further work seemed necessary.

The purpose of this investigation was to determine (1) by work on animals the toxicity of the protamine, salmine, (2) whether salmine can be administered safely to man, and (3) the dosage of salmine required to neutralize the anticoagulant effect of a standard dose of heparin in man.

*In vitro* studies demonstrated that 1.5 mg. of salmine neutralized 1.0 mg. of heparin, thus showing that there is quantitative neutralization. The lethal dose of salmine in the guinea pig was 6.0 mg. per 100 grams of body weight. Salmine injected intravenously in doses of 90.0 mg. per kilogram in the unanesthetized rabbit and 2.0 mg. per kilogram in the unanesthetized dog produced no reactions. The intravenous injection of salmine in doses of 10.0 mg. per kilogram in the anesthetized rabbit and 2.0 mg. per kilogram in the anesthetized dog produced transient drops in arterial blood pressure. No evidence of anaphylaxis was observed.

Intravenous injections of salmine neutralized heparin which was injected intravenously in dogs. In the dog, elevated coagulation times resulting from the intramuscular injection of heparin in the Pitkin menstruum were temporarily returned to normal levels by single intravenous injections of salmine.

The anticoagulant effect of intravenous injections of heparin in man was studied by determining coagulation times at fifteen- to thirty-minute intervals for a period of three hours. Coagulation times were determined by the Lee-White method with a thermostatically controlled water bath at 37° C. It was found that coagulation times were elevated four times the normal (four minutes) one hour after injection and that then they gradually returned to pre-heparin levels in three hours. With this to serve as a control, 50 mg. of heparin were injected intravenously into ten patients and fifteen minutes later salmine was injected intravenously in doses ranging from 15 to 50 milligrams. In the patients receiving 40 to 50 mg. of salmine, the elevated coagulation times were returned to normal levels within five minutes.

No toxic reactions were noted in any patient during or after the injections of salmine. It is concluded that the intravenous injection of 40 to 50 mg. of salmine promptly neutralizes the anticoagulant effect of 50 mg. of heparin and that such doses of salmine, when given slowly, produce no reactions.

#### 4. SURVIVAL OF TRANSFUSED SICKLE CELLS IN NORMAL SUBJECTS AND OF NORMAL RED BLOOD CELLS IN PATIENTS WITH SICKLE CELL ANEMIA

SHEILA T. E. CALLENDER, M.D.,\* AND JAMES F. NICKEL, A.B., ST. LOUIS, MO.  
(INTRODUCED BY CARL V. MOORE, M.D.)

This report describes observations made on (1) the survival of normal red blood cells transfused into patients with sickle cell anemia, (2) the survival of red blood cells from patients with sickle cell anemia transfused into normal subjects, and (3) the survival of red blood cells from persons with the sickle cell trait transfused into normal subjects. The Ashby technique of differential agglutination was used. By this method, the time of survival of normal cells in normal recipients has been found to be about 120 days; roughly one-half of the transfused cells disappear within 60 days. Results obtained confirm evidence indicating that the abnormality in sickle cell anemia is an inherent defect in the red blood cells and demonstrate a difference in behavior of erythrocytes from patients with sickle cell anemia and the sickle cell trait.

Normal red blood cells were transfused into three patients with sickle cell anemia. In all three, the survival of the donor cells was similar to that found in normal subjects. This suggests that there is neither a humoral factor in sickle cell anemia nor any element of increased phagocytosis which would accelerate destruction of normal cells.

Four reverse experiments were made using donors with sickle cell anemia and normal recipient subjects. The transfused cells disappeared rapidly from the circulation; nearly all donor erythrocytes were destroyed within thirty days. In one observation, one patient with sickle cell anemia served as a donor and another as the recipient; the transfused cells showed a comparable rapid destruction. A crude measure of the survival of sickle cells in the normal recipients was also made by study of sealed, moist preparations of capillary blood. Sickled donor cells could readily be demonstrated for only about three weeks after transfusion.

Blood from four healthy donors with the sickle cell trait was also given to normal subjects or to patients with mild hypochromic anemia. The survival of these cells was distinctly longer than that from donors with true sickle cell anemia. Four weeks after transfusion, at a time when cells from sickle cell anemia had largely disappeared from the normal recipients' circulation, more than one-half of the sickle cell trait erythrocytes were still circulating. Since these observations are currently in progress, the final survival rates cannot now be given.

The results obtained in this study confirm the evidence that there is an inherent defect in the erythrocytes of patients with sickle cell anemia which is responsible for the accelerated red cell destruction found in that disease. They also demonstrate that cells from subjects with the sickle cell trait have a longer survival time than cells from subjects with sickle cell anemia when transfused into normal recipient subjects. Whether this difference in behavior between sickle cell anemia and sickle cell trait cells is related to the ease with which sickling occurs or to some other qualitative difference in the erythrocytes has not yet been discovered.

\*Rockefeller Fellow from the Nuffield Department of Medicine, Oxford, Eng.

## 5. EFFECT OF EXTRINSIC FACTOR, LIVER EXTRACT, AND FOLIC ACID ON INDUCED MACROCYTIC ANEMIA OF SWINE

ROBERT W. HEINLE, M.D., ARNOLD D. WELCH, M.D. (BY INVITATION),  
WALTER L. GEORGE, M.D. (BY INVITATION), MARTIN EPSTEIN, M.D.  
(BY INVITATION), AND JACK A. PRITCHARD, M.D. (BY INVITATION)  
CLEVELAND, OHIO

Production of macrocytic anemia in a pig by administration of a folic acid-deficient diet with added crude folic acid antagonist has been described by Welch, Heinle, and colleagues. Subsequent studies show the anemia to be associated with megaloblastic hyperplasia of the marrow.

In a previous report such an animal was shown to obtain a hematologic as well as very evident clinical response following the administration of a source of extrinsic factor together with normal human gastric juice. The use of the latter was probably unnecessary, as demonstrated by later experiments. These animals do not develop histamine-fast achlorhydria.

Following the described response, the same pig was allowed to relapse and was given a single injection of 15 units of purified liver extract. This was followed by another hematologic and clinical response.

Another pig with induced anemia received four daily intramuscular injections of 10 mg. of folic acid. This was followed by rapid and marked hematologic and clinical improvement.

A third pig developed severe anemia and neutropenia and became critically ill, appearing to be in a moribund state. Administration of two intramuscular injections of 15 units of purified liver extract, 10 mg. of folic acid, and 35 mg. of niacinamide caused rapid and marked hematologic and clinical improvement.

Bethell and co-workers and Welch and co-workers have offered evidence that a substance (s) in purified liver extract may contribute to the proper utilization of conjugates of folic acid by patients with pernicious anemia in relapse. Results obtained in pigs, however, indicate that extrinsic factor and purified liver extracts are effective even though folic acid is not available to the animal.

Folic acid and its conjugates are not in themselves extrinsic factor as evidenced by the facts that folic acid is effective in pernicious anemia without added intrinsic factor and, further, materials free of demonstrable folic acid or its conjugates may be potent sources of extrinsic factor.

It has been demonstrated by several investigators that purified liver extracts effective in pernicious anemia are devoid of significant amounts of folic acid.

In view of recent experimental data, Welch has proposed that folic acid and the antipernicious anemia principle of liver may function via unrelated chemical pathways. While one is reluctant to believe that these substances have no direct or sequential relationship, it can be stated that data available at this time fail to support the idea of such relationship.

## 6. FURTHER STUDIES OF THE HYPERSPLENIC MECHANISM IN HUMAN DISEASE SYNDROMES

CHARLES A. DOAN, M.D., AND CLAUDE-STARR WRIGHT, M.D.  
COLUMBUS, OHIO

Hypersplenic syndromes may present as acute surgical emergencies or as chronic invalidism and may involve the red cells, and/or neutrophils, and/or thrombocytes. They may reflect pathology inherent and primary in the spleen or secondary to some generalized systemic disease. Splenectomy is the only satisfactory treatment to date.

*Diagnosis* is dependent upon (1) an adequate history and supporting physical findings (with or without gross splenic enlargement); (2) peripheral cytopenia; (3) sternal marrow aspiration showing normal maturation with compensatory cellular hyperplasia of all deficient circulating elements; (4) an adrenalin test "biopsy" to reveal splenic hypersequestration of all involved cell strains.

The *mechanism* resulting in more or less profound peripheral cytopenia probably reflects both cellular and humoral factors: Parenchymal cellular hypersequestration + excessive concentrations of lysolecithin (or some similar lytic enzyme or enzymes) + R-E cell hyperplasia and hyperphagocytosis = hemolytic anemia, neutropenia, and/or thrombocytopenia. That the hyperplastic bone marrow is actually, as well as apparently, functionally effective in its delivery of essential elements may be seen (1) by immediate direct studies of living marrow cell preparations and (2) by studies at the operating table of splenic artery and vein blood before and after adrenalin induced contraction of this organ. As many as 350,000 platelets have been discovered entering the spleen with only 17,000 leaving it, whereas upon adrenalin introduction via the splenic artery the splenic vein and peripheral capillaries promptly show numbers of platelets leaving the spleen comparable to those carried by the artery. Furthermore, supravital scrapings of the freshly removed spleen show direct microscopic evidence of excessive R-E cell hyperplasia and hyperphagocytosis. In nine pathologic spleens removed for hypersplenism a quantitative increase of lecithinase activity was demonstrated by Scheff and Awny in our laboratory in sharp contrast with two normal control spleens.

Certain clinical and hematologic *recurrences* of hypersplenism may be caused by overlooked accessory splenic tissue, or by its subsequent hypertrophy, or by accidentally implanted "splenosis" fragments. Usually the original hematologic syndrome is repeated, but not necessarily. In one of our series of 216 patients diagnosed on the basis of a hypersplenic mechanism the original problem was that of hemolytic anemia. Splenectomy promptly and permanently alleviated this acute critical danger. Eighteen months later a thrombocytopenic crisis developed and surgical re-exploration, after an appropriate diagnostic study, revealed a 5 gram accessory spleen (extraperitoneal in location at the lower pole of the left kidney), the removal of which completely, and promptly, and permanently restored the platelet equilibrium to normal with cessation of all purpuric manifestations. Recurrences have also been found on surgical re-exploration to be due to a generalized R-E cell hyperplasia and hyperphagocytosis involving liver, lymph nodes, and bone marrow, in which case symptomatic therapy only is available.

The pathologic tissue *classification* recognized in our series of patients covering the past seventeen years, as studied by von Haam and Awny, are:

PRIMARY			
Congenital hemolytic icterus	72	Recurrences	
Thrombocytopenic purpura	62	Accessory spleens	3
Splenic neutropenia	11	Generalized R-E cell hyper-	3
Splenic panhematopenia	10	phagocytosis	
SECONDARY			
(1) Congestive splenomegaly		(4) Inflammatory splenomegalies	
Banti's syndrome	36	Tuberculosis	1
Felty's syndrome	3	Syphilis	1
Acquired hemolytic icterus	6	Moniliasis	1
(2) Infiltrative splenomegaly		Boeck's sarcoid	1
Gaucher's disease	3	Hodgkin's syndrome	3
Xanthomatosis	1	(5) Neoplastic splenomegaly	
(3) Hemoblastic splenomegaly		Retothelio sarcoma	1
Lymphatic leukemia	1	Hemangioma	2
Myelogenous leukemia	1		

The *hypersplenic mechanism*, as now conceived, is an adequate explanation for a rather wide range of human clinical and hematologic syndromes, hitherto considered to be distinct and sharply separated etiologic entities.

## 7. THE PRESIDENT'S ADDRESS

### 8. PROGRESS REPORT ON A STUDY OF VASCULAR CHANGES IN THE SKIN IN PREGNANCY

WILLIAM B. BEAN, M.D., MORRIS W. DEXTER, M.D. (BY INVITATION), ROBERT C. COGSWELL, M.D. (BY INVITATION), AND JAMES EMBICK, M.D. (BY INVITATION), CINCINNATI, OHIO

As a continuation of investigations on vascular spiders and palmar erythema in chronic disease of the liver, we have observed similar phenomena in 1,200 pregnant women, 278 of whom have been followed during pregnancy and for at least six weeks after delivery. In the smaller group observed during and after pregnancy there were 207 Negro women and 71 white women. Vascular spiders have been found in 60 per cent of the white women and only 15 per cent of the Negroes, while palmar erythema was found more frequently (49 per cent) in white than in Negro women (31 per cent). These signs tend to appear during the first two trimesters of pregnancy but may originate during the last months of pregnancy. In a control group of nonpregnant women vascular spiders were found in 16 per cent of the white women and none of the Negro women, while palmar erythema was noted in 5 per cent of the white and 4 per cent of the Negro women.

Both vascular spiders and palmar erythema tend to fade at the time of delivery and in approximately 80 per cent of the cases the lesions can no longer be detected six weeks after delivery. We have found no significant difference in disappearance rate between white and Negro women. Analysis of age, season, parity, sex and viability of child, toxemia, and other factors has revealed no special relation to the incidence of vascular changes in the skin. Liver function tests during and after pregnancy have not been significantly different in those with vascular spiders, palmar erythema, or both, and those without the lesions. Studies of the excretion of steroid hormones is in progress, but data are not yet adequate for evaluation.



## 9. HEREDITARY HEMORRHAGIC TELANGIECTASIA IN ASSOCIATION WITH CEREBRAL MANIFESTATIONS AND PULMONARY ARTERIOVENOUS ANEURYSM

CHARLES F. WILLIAMS, M.D. (By INVITATION), ST. PAUL, MINN., AND  
EDMUND B. FLINK, M.D., MINNEAPOLIS, MINN.

Within the last year two cases of hereditary hemorrhagic telangiectasia or Osler's disease were encountered on the medical service of the Ancker Hospital. Both patients presented findings which satisfy the diagnostic criteria of Osler's disease. Both had a family history of repeated epistaxis in several members and in two generations. Both had small spiderlike angiomas on the face and neck and some punctate angiomas on the skin and mucosa of the mouth and nose which bled with arterial spurting. The first patient was anemic at death undoubtedly from chronic blood loss. The second patient had cyanosis and mild polycythemia because of a pulmonary arteriovenous aneurysm. The first patient, who was mentally deficient, had been observed from the age of 32 years at the Ancker Hospital. He had never had hypertension. At the age of 37 years he had an episode of hemiplegia, after which he continued to have fainting spells and hemiparesis. At the age of 46 years he was hospitalized because of sudden temporary loss of consciousness. Eight days later he became comatose and died in twenty-four hours. No autopsy was obtained, but it is probable from clinical evidence that he had suffered an intracerebral or subarachnoid hemorrhage. The second patient had also been admitted to the Ancker Hospital at the age of 30 years because of convulsions and unconsciousness. A diagnosis of idiopathic epilepsy was made. He had no more convulsions until the age of 33, at which time he was hospitalized because of a scalp injury sustained during a convulsion. On auscultation of the chest one could hear a systolic murmur in the second left interspace anteriorly. The murmur was accentuated by inspiration. Fluoroscopy revealed a pulsating mass in the left upper lobe. This mass expanded on deep inspiration and contracted noticeably on the Valsalva maneuver. The effects of respiration and the Valsalva maneuver were recorded by planograms. An electroencephalogram was interpreted as being in keeping with an ordinary epilepsy and was not focal in type. Goldstein has reported a case similar to the first patient. Of the fourteen patients with pulmonary arteriovenous aneurysms reported on, eight seem to have had Osler's disease also, but only one was reported as such. Patients with pulmonary arteriovenous aneurysm reported on have had epilepsy, vertigo, syncope, and attacks of numbness and paresthesias of unexplained variety.

*Summary.*—Two cases of Osler's disease are presented. Both of the patients had cerebral manifestations and one of them also had a pulmonary arteriovenous aneurysm.

## 10. FACTORS INFLUENCING PRESSURE IN THE PORTAL VEIN AS MEASURED IN THE INTACT ANIMAL

F. W. HOFFBAUER, M.D., J. L. BOLLMAN, M.D., AND J. H. GRINDLAY, M.D.  
(By INVITATION), ROCHESTER, MINN.

The influence of various physiologic factors upon the pressure in the portal vein has been studied in the intact nonanesthetized dog. In the past, most determinations of portal vein pressures have been limited to measurements made in the anesthetized animal during operation. Information as to the effect of exercise, of ingestion of food, and of straining has not been available. A knowl-

edge of such influences may have clinical significance in relation to the problem of portal hypertension. The problems presented by the existence of such collateral venous channels as the gastric and esophageal varicosities in cirrhosis may, in some cases, outweigh the problem presented by the hepatic metabolic alterations.

#### METHOD

A 24-inch length of flexible polyvinyl chloride plastic tube\* of small bore (internal diameter, 2 mm.) is inserted into a branch of the splenic vein at operation. Before the tube is tied in place, it is threaded down the splenic vein until the tip is just within the portal vein. The tube is led to the outside through a stab wound. In order to record changes in intraperitoneal pressure, a thin rubber balloon (capacity, 60 ml.) is placed in the peritoneal cavity. The balloon is filled with water and connected to a similar length of plastic tubing. Pressure in the inferior vena cava has also been determined by a technique similar to that used for the portal vein. A left nephrectomy is performed. A plastic tube is inserted through the renal vein.

The venous cannulae are filled with a citrate solution and the free ends are plugged when readings are not being made. This plastic tubing causes much less reaction in the lumen of the vein than does rubber or glass. Thrombus formation may be delayed by the administration of dicumarol. A free flow of blood from the tube may be obtained for ten to fourteen days, occasionally longer. The time interval is sufficient for complete recovery from the effects of the operation before observations are undertaken. Pressure readings are made in water manometers attached to the side of the dog by means of a light harness. All readings are made with the animal in the standing position.

#### RESULTS

In normal intact dogs the pressure in the portal vein varies from 4 to 10 cm. of water. The pressure in the inferior vena cava is 0, or occasionally 2 cm. below atmospheric pressure. Exercise produces no significant increase in portal pressure. The straining that accompanies urination, defecation, and vomiting produces sharp increases in the portal pressure. This is associated with increases in intra-abdominal pressure and in inferior vena caval pressure as well.

The ingestion of food produces an increase in portal vein pressure that persists for several hours. Initially this is associated with a rise in intra-abdominal pressure. The latter returns to the original level before the portal vein pressure does. There is a period following the ingestion of food during which the elevation of portal pressure appears to be unopposed by other forces.

Acute liver damage resulting from exposure to carbon tetrachloride vapor does not appear to be associated with a portal hypertension despite the fact that such livers appear congested upon examination. Such animals have shown a normal portal vein pressure response during exercise.

It is hoped that observations by this method in the intact animal may prove useful in studying the relationship of portal vein pressure to development of collateral venous channels and to ascites formation. It should also be possible to evaluate the reduction of portal pressure brought about by surgically created venous shunts in experimental cirrhosis.

\*The plastic tubing used in these experiments is manufactured under the trade name "Transflex" and was furnished by the courtesy of Irvington Varnish and Insulator Company, Irvington, N. J.

## 11. TREATMENT OF ENDOGENOUS HYPOVITAMINEMIA A IN LIVER DISEASE

HANS POPPER, M.D., FREDERICK STEIGMANN, M.D., AND HATTIE A. DYNIEWICZ,  
PH.C. (BY INVITATION), CHICAGO, ILL.

Previous experiments have shown that the vitamin A metabolism in liver disease is altered in several ways. The release of vitamin A from the liver is disturbed since in acute liver disease, despite considerable hepatic vitamin A stores, the plasma vitamin A drops rapidly. This disturbance in vitamin A release is due to displacement of vitamin A within the liver cells as demonstrated by fluorescent microscopy. Another disturbance is impaired intestinal absorption as indicated by the flat vitamin A tolerance curve after oral administration of vitamin A. The endogenous nature of the hypovitaminemia A in liver disease points, therefore, to the necessity of administering extremely large doses of vitamin A or to correction of the faulty intestinal absorption. Administration of bile acids did not alter the flat tolerance curve.

In more recent experiments the administration of lecithin, wheat germ oil, and a fat-soluble antioxidant did not significantly or regularly raise the tolerance curve in hospital control subjects or in patients with liver disease. The administration of 50 mg. of mixed tocopherols simultaneously or for several days prior to the administration of 75,000 units of vitamin A also did not raise the curve significantly. This was confirmed by experiments on vitamin A-deficient rats in which the hepatic vitamin A concentration after feeding of one dose of vitamin A was not influenced by the simultaneous administration of tocopherol. In contrast, the hepatic vitamin A concentration after administration of repeated doses of vitamin A is far higher in animals receiving supplements of tocopherol. This points to a protecting mechanism of the latter within the liver.

Following recent reports about improved absorption of aqueous vitamin A preparations, the vitamin A tolerance curve was compared after administration of vitamin A in oil and in aqueous solution containing sorbitan monolaurate derivative as dispersing agent and propyl gallate as antioxidant. In twenty hospital control subjects and thirty-six patients with various liver disease (most of them with low plasma vitamin A), the tolerance curve after administration of 30,000, 75,000, or 600,000 units of vitamin A was much higher with the aqueous than with the oily solution, independent of the order of administration. The flat vitamin A tolerance curve in liver disease was thus raised to peaks above those found in hospital control subjects after administration of equal amounts in oil. The administration of aqueous vitamin A preparations therefore appears suitable for compensating the faulty absorption of vitamin A caused by liver damage.

## 12. STUDIES IN NITROGEN METABOLISM

D. KOZOLL, M.D., AND W. T. MOK, M.D. (BY INVITATION)  
CHICAGO, ILL.

*Influence of Oral and Intravenous Administration of Amino Acids Upon Nitrogen Balance.*—A varied opinion exists as to the relative effectiveness of amino acids given by mouth and by vein, most workers indicating a slight advantage of the latter. Our clinical experience differed, and for that reason a study was carried out in which eight patients were given identical amounts of the same amino acid preparation by vein and by mouth and the nitrogen balance achieved compared. Each patient was kept on a 2,000 calorie diet which

was virtually nitrogen free for the entire period of study. For periods of five days each the patients were given quantities of a lyophilized amino acid preparation\* intravenously and then the same quantity was given for another five-day period orally. In three of eight patients, oral administration produced a positive balance at the level of amino acids given, whereas the same quantity intravenously did not. In seven of the eight patients, the nitrogen balance during periods of oral administration was significantly higher than during equivalent periods of intravenous use. In one patient, in whom both routes produced positive balance, that produced by the intravenous route was significantly higher. This evidence indicates the advantages of the enteral route of administration of protein digests wherever possible.

*Influence of Caloric Intake Upon Nitrogen Balance.*—There has been established a concept that the degree of nitrogen balance will be greatly influenced by the caloric intake. Actually, few studies have investigated this particular point. In five patients a constant quantity of lyophilized amino acid preparation† was administered intravenously daily for five periods; this dose was calculated barely to approach nitrogen equilibrium. During each five-day period, the oral caloric intake, which consisted of a carbohydrate diet, almost free of protein, was varied between 500 and 3,000 calories. It was found necessary to administer a minimum of approximately 1,000 calories per day during each period to produce nitrogen balance. However, in no instance did an increase in the caloric intake beyond 1,000 calories lead to a consistently more positive nitrogen balance. This supports previous observations in which no consistent correlation could be made between the caloric intake of a given patient and the nitrogen balance achieved.

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\*Provided by the Interchemical Corporation and prepared for intravenous use.

†Interchemical Corporation.

## PROGRAM

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SCIENTIFIC PROGRAM—OCTOBER 31, 1947

FRIDAY AFTERNOON, 2:00 P.M.

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### 13. PENICILLIN IN THE TREATMENT OF ACTINOMYCOSIS

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ROCHESTER, MINN.

An appraisal of the value of penicillin in the treatment of actinomycosis is reported. The report is based on a review of ninety-eight cases of maxillo-facial, pulmonary, abdominal, and pelvic infection. The period of follow-up of these patients varied from one year to five years. Forty-five patients received penicillin; fifty-three patients did not receive it.

Of the group of patients suffering from maxillofacial actinomycosis, twenty-six received penicillin and twenty-five did not. Examination of the results reveals that the number who recovered was approximately equal in both groups (in excess of 90 per cent). However, the period of disability of those patients who received penicillin was markedly shortened. Furthermore, the sinuses healed within a short period after the institution of penicillin therapy.

From the standpoint of comparison between those patients who received penicillin and those who did not, the most striking results occurred in the pulmonary, abdominal, and pelvic infections. Nine patients suffering from pulmonary actinomycosis received penicillin. There were five recoveries and four failures. Of the thirteen patients suffering from pulmonary actinomycosis who were untreated or received treatment other than penicillin, seven died, five still had evidence of disease when last heard from, and only one showed evidence of improvement. Of seven patients with abdominal actinomycosis who were treated with penicillin, five recovered and two did not. Of fourteen patients suffering from abdominal actinomycosis who did not have the benefit of penicillin therapy, nine died, four still had evidence of disease when last heard from, and only one was said to have recovered. There were three patients suffering from actinomycosis involving the pelvic viscera, all of whom recovered after surgical drainage and penicillin therapy. There was one patient with pelvic actinomycosis in whom penicillin was not used. The treatment employed was not effective. Prior to the introduction of penicillin therapy, instances of pelvic actinomycosis in which cures were obtained were, in our experience, rarely if ever encountered.

The diagnosis of actinomycosis in each case was made by means of direct examination or culture of pus or of material obtained at operation. All strains of *Actinomyces bovis* cultured were found to be sensitive to the action of penicillin in vitro.

#### 14. EFFECT OF PULMONARY EDEMA ON SUSCEPTIBILITY OF MICE TO PNEUMOCOCCAL PNEUMONIA

CARL G. HARFORD, M.D., AND MARY N. HARA, A.B. (BY INVITATION)  
St. Louis, Mo.

Previous experiments have demonstrated that type I pneumococci are eliminated rapidly from the lung of the normal mouse but grow in the lung containing an influenza viral lesion. Histologic examination of the influenza viral lesion in the mouse has shown the presence of intra-alveolar fluid. The object of the present experiments was to determine whether this fluid could serve to explain the observation of growth of pneumococci in the viral lesion.

Advantage was taken of the facts determined by others, that in the normal animal, protein-containing fluids are removed slowly from the alveoli while crystalloids are absorbed within a few minutes. In confirmation of this slow removal, we have demonstrated in tissue sections that normal mouse serum instilled into the alveoli of normal mice will persist there for at least five hours.

Accordingly, normal serum or saline was given by intrabronchial cannula to groups of mice which then were allowed to inhale fine droplets of type I pneumococci and were observed for survival. In another experiment, the pneumococci were suspended in the inoculum of serum or in Locke's solution for control and the number of pneumococci in the left lobe of the lung determined three hours later in poured blood agar plates.

The results of all experiments indicated that the presence of serum in the alveoli rendered the mice more susceptible to fatal pneumococcal infection and interfered with the normal mechanism for elimination of pneumococci.

The manner of action of serum in the alveoli seems likely to be a combination of three factors: (1) interfering with surface phagocytosis, (2) providing a means of spread of pneumococci throughout the lung in the same way as the edema fluid of established pneumococcal pneumonia, and (3) supplying a culture medium for bacterial growth.

The experiments give evidence supporting the theory that the presence of alveolar fluid is an important factor in the mechanism by which the influenza viral lesion lowers resistance of the lung to secondary bacterial infection. The results suggest also that pulmonary edema in man due to any cause may bring about a greater susceptibility to pathogenic bacteria and pneumonia.

#### 15. SURFACE PHAGOCYTOSIS OF TYPE III PNEUMOCOCCUS

W. BARRY WOOD, JR., M.D., AND MARY RUTH SMITH, M.S. (BY INVITATION)  
St. Louis, Mo.

Surface phagocytosis, a defense reaction of the host unrelated to humoral immunity, has recently been shown to play an important role in the mechanism of recovery in acute bacterial pneumonia. Since type III pneumococcus produces a particularly virulent form of pneumonia, its behavior toward leucocytes has been studied both *in vitro* and in laboratory animals. Although experimental type III pneumonia in rats has been found to resemble type I pneumonia both in regard to pathogenesis and response to chemotherapy, it differs from the latter in that it frequently causes multiple lung abscesses. Also certain strains of type III pneumococcus, when given to mice via the respiratory tract, are less effectively destroyed in the lung and cause a significantly higher fatality rate.

A plausible explanation for the greater virulence of these type III pneumococci is suggested by the fact that they are more resistant to surface phagocytosis than is the type I organism. Evidence has been obtained that the increased resistance to surface phagocytosis is related to the presence of a peculiar outer capsule or "slime layer" possessed only by the highly virulent strains of pneumococcus III. The slime layer stains metachromatically with methylene blue and is present only on the surfaces of young, rapidly multiplying organisms. When the slime layer is lost with age, the bacterial cell loses its resistance to surface phagocytosis.

Of particular interest is the fact that pneumococcus type III loses its slime layer *in vivo* during chemotherapy. Once the outer layer is lost, the type III organism, like other encapsulated bacteria, is phagocyted by the surface mechanism and is ultimately killed and digested by the phagocytic cells. These findings would appear to explain certain well-established clinical observations concerning type III pneumococcal infections, particularly in regard to virulence and response to chemotherapy.

#### 16. ANTIHYALURONIDASE ACTIVITY IN HUMAN SERUM WHICH INACTIVATES THE HYALURONIDASES OF TYPES I, II, AND VII PNEUMOCOCCI: RISE IN TITER FOLLOWING PNEUMOCOCCUS BACTEREMIA

ROBERT T. THOMPSON, M.D., CINCINNATI, OHIO  
(INTRODUCED BY M. A. BLANKENHORN, M.D.)

A preliminary report on this subject was read at these proceedings two years ago. At that time rises in titer of antihyaluronidase activity which antagonized the hyaluronidases of types I, II, and VII pneumococci were reported in serial sera of five patients following pneumococcus bacteremia. These rises in titer occurred eight to twenty days after bacteremia and were not type specific. Further work on this problem, using the mucoprotein clot prevention test (McClean, 1943) as before, is reported here.

Including patients in the previous report, the serial sera of twenty-six patients with primary pneumococcal pneumonia had initial serum taken within three days of admission to the hospital. Results in these patients are reported here to indicate the occurrence of antihyaluronidase activity in the course of pneumococcal pneumonia.

None of seven patients with negative blood culture exhibited more than twofold rise of antihyaluronidase titer. Serial sera of two patients were titrated against the three enzymes, serial sera of four patients were titrated against two enzymes, and serial sera of one patient were titrated against one enzyme. Changes of antihyaluronidase titer found in these fifteen titrations were one-fourth-fold once, one-half-fold four times, no change five times, and twofold five times.

Eight of eleven patients with pneumococcus bacteremia and no purulent complication exhibited rises of antihyaluronidase titer which were fourfold or greater. Serial sera of all these patients were titrated against the three enzymes. Rise of antihyaluronidase titer found in these thirty-three titrations were no change once, twofold seven times, fourfold six times, eightfold eleven times, and sixteenfold eight times.

All of eight patients with pneumococcus bacteremia and purulent complications exhibited rises of antihyaluronidase which were fourfold or greater. Serial sera of all these patients were titrated against the three enzymes. Rises of antihyaluronidase titer found in these twenty-four titrations were fourfold six times, eightfold five times, sixteenfold three times, thirty-twofold three times, and sixty-fourfold or greater seven times.

One patient with mesothelioma of pleura complicated by pneumococcal empyema and another patient with pneumococcal meningitis secondary to otitis media had rises of antihyaluronidase titer.

These rises of antihyaluronidase titer probably signify excessive elaboration of hyaluronidase by the invading pneumococci, since the antigenicity of hyaluronidases has been demonstrated by McClean (1943). Hyaluronidases act as spreading factors (Chain and Duthie, 1939) so that this elaboration of hyaluronidase may explain the excessive morbidity and mortality of bacteremic pneumococcal pneumonia.

## 17. EXPERIMENTAL AND CLINICAL INVESTIGATIONS ON THE SPECIFIC TREATMENT OF HUMAN BRUCELLOSIS\*

WESLEY W. SPINK, M.D., WENDELL H. HALL, M.D. (BY INVITATION), JAMES SHAFFER, M.D.† (BY INVITATION), AND ABRAHAM I. BRAUDIE, M.D. (BY INVITATION), MINNEAPOLIS, MINN.

The fundamental requisite of successful therapy in human brucellosis is that brucella should be eradicated from the tissues. With this principle in view, this report summarizes a series of investigations which have been under way in the laboratories and clinics of the University of Minnesota Hospitals during the past ten years.

An excellent method for rapidly screening potential therapeutic agents has utilized fertilized chicken eggs infected with a virulent culture of *Brucella abortus* via the yolk sac. Uniformly lethal infections were established in seven-day embryos. With the inoculum used, 50 per cent of untreated controls were dead six days after infection, and 100 per cent, nine days after infection. When sulfadiazine was introduced into the sac twenty-four hours after the brucella, 50 per cent survived twelve days or more after infection. When streptomycin was injected, 50 per cent of the embryos survived nine to eleven days. When the embryos were treated with a combination of sulfadiazine and streptomycin, 50 per cent survived nine to eleven days. An important feature of these observations was not only the protection afforded by these agents, but also the results of cultural studies of the tissues. Brucella were recovered from all untreated controls. Sulfadiazine-treated embryos, when sacrificed, revealed brucella present in 90 per cent, while 77 per cent of the streptomycin-treated eggs contained brucella. Embryos receiving sulfadiazine and streptomycin showed brucella present in the tissues in only 13 per cent.

The foregoing data have correlated remarkably well with the results of therapy in patients having active brucellosis. Since an evaluation of treatment in this disease is dependent upon bacteriologically proved cases, studies have been carried out on thirty-five individuals from whose tissues or body fluids

\*Aided by a grant from the United States Public Health Service.

†Research Fellow in Chemotherapy under a grant from Commercial Solvents Corporation.



brucella have been isolated. *Br. abortus* was recovered from thirty-three patients and *Brucella melitensis* from two. Sulfonamides, especially sulfadiazine, were used alone in twenty patients, and coincident with treatment complete recovery occurred in only six patients. Seven patients were treated with streptomycin and the results were less satisfactory than those obtained with sulfadiazine. Nine patients have been treated simultaneously with sulfadiazine and streptomycin with quite satisfactory results. One patient with subacute bacterial endocarditis due to *Br. abortus* recovered completely, except for a residual vertigo, and has remained well for four months after treatment. One patient with a severe and disabling spondylitis recovered promptly. The optimum dose of the two agents awaits further studies, but at the present time it is recommended that 0.5 Gm. of streptomycin should be injected intramuscularly every six hours for seven days for a total of 14 grams. Therapy with sulfadiazine is started at the same time with an initial oral dose of 4 Gm. and then 1 Gm. every four hours for two to three weeks. In conclusion, experimental and clinical investigations indicate that the simultaneous use of streptomycin and sulfadiazine will provide a more satisfactory form of specific therapy for brucellosis than has been described to date.

## 18. SIGNIFICANCE OF COPROANTIBODY EXCRETION DURING ENTERIC INFECTION\*

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(INTRODUCED BY JAMES A. GREENE, M.D.)

A study was made of fecal antibody excretion from patients with salmonella and shigella infections. Diarrheal and dysenteric stools obtained during the acute infection, recovery, and convalescence were examined for coproantibody simultaneously with culture and serum agglutinin titration. Positive cultures were obtained from 58.6 per cent of the patients and fecal agglutinins were found in 91.3 per cent. Coproantibody for the homologous organism, or for one or more enteric pathogens in those specimens from which a positive culture was not obtained, was demonstrated in high titer on several occasions from each patient. The antibody was present as early as the third day, reached peak titer by the tenth day, rapidly declined as recovery was initiated, and completely disappeared during convalescence. In those cases in which clinical recovery was not obtained, coproantibody persisted throughout the observation period. Circulating antibody did not rise to significant titer until about the time recovery was initiated and then gradually rose to high titer which persisted during the remainder of the observation period. Coproantibody titration provided a measure of efficacy of therapy in that following a good chemotherapeutic response coproantibody titer rapidly declined and disappeared when cultures became negative and after recovery definitely was established. In contrast, coproantibody persisted when clinical recovery was not obtained.

Coproantibody could be demonstrated only during active infection, or clinical activity of disease, and was not present in carriers who showed no evidence of infection. In chronic ulcerative colitis coproantibody for one or more species of shigella, and often for species of salmonella, was present during episodes of exacerbation of symptoms.

\*Aided by a grant from the Committee on Scientific Research of the American Medical Association.

## 19. RESULTS OF VACCINATION AGAINST EPIDEMIC INFLUENZA DURING THE SPRING OF 1947

CLAYTON G. LOOSLI, M.D., PH.D., JAMES A. SCHOENBERGER, M.D. (BY INVITATION), AND G. BARNETT, M.S. (BY INVITATION), CHICAGO, ILL.

In the fall of 1946, a study was organized to test further the prophylactic value of vaccination against epidemic influenza. Two thousand twenty students living in university houses were employed. Seven hundred ninety were vaccinated and 1,230 served as control subjects. Three different commercial influenza vaccines, containing 50 per cent Lee, 25 per cent PR-S, and 25 per cent Weiss strains and prepared by the red cell eluate, calcium phosphate adsorption, and high speed centrifugation methods, respectively, were used. The number of students receiving the three vaccines was approximately the same. Vaccinations, given in the dormitories, were begun November 6 and completed December 6, 1946. The immune response to the three vaccine preparations was approximately the same as determined by the Salk modification of the Hirst chicken red cell inhibition agglutination test.

A sharp increase in the incidence of acute respiratory disease characteristic of mild influenza occurred among the student population during the first week of March, 1947. Antibody determinations for influenza A virus (PR-S and Weiss strains) showed an increase in titer in convalescent sera from a portion of the unvaccinated patients admitted to the hospital. During the period of increased incidence (March 2 to April 5, 1947), the attack rate was the same (9.5 per cent) in the vaccinated (seventy-five) and unvaccinated (117) groups, with 2.5 per cent (twenty) and 2.36 per cent (twenty-nine), respectively, being admitted to the hospital.

Two strains of influenza virus, found to be closely related, were isolated from throat washings employing the egg technique. The sera of some of the patients admitted to the hospital, both from the vaccinated and unvaccinated groups, showed a marked rise in antibody titer against these strains. Antibody titers for influenza A virus (PR-S) in the acute sera of patients admitted from the vaccinated group were only slightly lower than the postvaccination levels. Sixty pairs of pre- and postvaccination sera were again tested for antibodies against the PR-S, Weiss, and Lee strains of virus as well as the new strains. There was an average of fourfold or greater rise in titer against the former and none against the latter. This shows that there was no close antigenic relationship between the new strains causing the epidemic and those making up the vaccine. This would explain the lack of effect of the vaccine in this outbreak of influenza. The problem of control of epidemic influenza in the light of the foregoing findings is discussed.

## 20. THE ROLE OF THE ADRENAL GLAND IN IMMUNE MECHANISMS

BENJAMIN C. HOUGHTON, M.D., J. S. THATCHER, M.D., AND  
CAROLYN HILLES, COLUMBUS, OHIO

(INTRODUCED BY C. A. DOAN, M.D.)

Interest in the relation of the adrenal cortical hormone to the development and release of antibody globulin has been revived in recent years by the work of Dougherty, White, and associates. These authors have reported producing a remarkable augmentation of circulating antibody titers by the administration of

single large doses of adrenal cortical hormone to immunized animals. The anamnestic effect of this hormone exceeded, but was comparable to, the increase in immune titers induced by nonspecific protein and specific antigen administration. Since pituitary adrenotrophic hormone was also effective in this respect, it was suggested that the release of antibodies is a phenomenon mediated by the activity of the adrenal cortex. The concurrent reduction in total lymphatic tissues observed in treated animals led to the conclusion that the dissolution of lymphocytes effected by the concentration of adrenal hormone yields antibody globulin to the component proteins of plasma.

The question advanced by this work relates to the effect of the adrenal cortical function on the (1) formation, (2) release, or (3) distribution of antibody globulin. For the purpose of studying the hormonal effects upon the formation of immune bodies, two groups of cats were employed as experimental animals. One group was given suspended, washed sheep erythrocytes intravenously. The other group was adrenalectomized, maintained on 1 mg. of DOCA daily until adequate equilibrium was established, and then immunized against sheep erythrocytes in an identical manner. Titers of immune body developed in both groups without significant difference. Symptoms of anaphylaxis were exhibited by both groups upon re-exposure to antigen. Sodium ion levels were maintained without change in the adrenalectomized animals throughout the experimental period.

To study the effect of cortical hormone on the release or distribution of antibody globulin, three groups of immunized animals were used. These consisted of normal rabbits, normal cats, and adrenalectomized cats maintained on desoxycorticosterone acetate. Each group was subjected to a large single intravenous injection of whole adrenal cortical extract (Thatcher and Hartman). No augmentation of titers was observed at three, six, nine, or twelve hours, or subsequently. Realizing the possible differences in preparation, refinement, and content of hormone, this experiment was repeated using Wilson's whole cortical extract. Results were identical.

It is apparent, therefore, that the adrenal cortical hormones do not either affect the capacity of the animals to form immune bodies when exposed to antigen nor alter the titers of such immunity when it is established.

Hematologic studies carried out concurrently on normal immunized and adrenalectomized cats revealed wide individual variations in total lymphocyte levels. These levels, high or low, bore no relation to the capacity to form or maintain titers of immunity.

## 21. INSULIN DEPRESSION AND CARBOHYDRATE EXCITATION OF THE PANCREATIC ISLETS OF LANGERHANS

JEROME W. CONN, M.D., AND STEFAN S. FAJANS, M.D. (BY INVITATION)  
ANN ARBOR, MICH.

Patients suffering from periodic spontaneous hypoglycemia produced by secreting islet cell tumors do not all yield the same blood sugar response to a test dose of glucose. About one-half of such patients have responded with a hyperglycemic, plateau (diabetic) type of curve, while the others have shown a normal or subnormal initial elevation of the blood sugar, followed by a rapid fall to hypoglycemic levels. Fasting hypoglycemia has been common to both groups. The discrepancy has aroused abundant speculation.

Our recent studies upon such patients have suggested that: (1) The presence or absence of the diabetic type of curve is dependent upon the functional

state of the islets of the pancreas proper. (2) The functional state of the pancreatic islets is conditioned by the intensity of the hyperinsulinism produced by the abnormal tissue; the more severe the hyperinsulinism, the more likely is pancreatic depression and a diabetic type of curve. (3) The amount of carbohydrate in the antecedent diet exerts an important influence upon the response.

The present experiments were designed to test these tentative conclusions. Dogs on constant diets were chronically hyperinsulinized with large daily doses of protamine zinc insulin for periods of four to eight months. Daily fasting blood sugar was maintained between 20 and 40 mg. per cent. Four-hour glucose tolerance tests were done weekly under a variety of conditions. The same animals were then made diabetic with alloxan, again hyperinsulinized as before, and the same experiments repeated. Differences in results before and after alloxan can be attributed to the presence or absence of the beta cells of the islets of Langerhans.

The results indicate:

1. The secretion of insulin by the pancreas can be greatly inhibited by long-continued administration of exogenous insulin, but sufficient insulin to produce severe, chronic hypoglycemia is required. Under these circumstances the blood sugar response to a test dose of glucose is of the diabetic type, even though there is sufficient steadily absorbed insulin acting during the test to have produced intense and prolonged hypoglycemia when no glucose was given over a similar test interval.

2. A small amount of insulin given intravenously at the height of the diabetic response to glucose produces a precipitous fall of the blood sugar. Thus the ability of the animal to respond normally to a sharp rise of the blood insulin level remains intact.

3. Insulin-inhibition of endogenous insulin production can be overcome in a few days by the administration of large amounts of carbohydrate despite continuation of the inhibiting dose of insulin. The hypoglycemic phase of the tolerance curve is restored.

4. Following alloxan destruction of the beta cells and the reinstitution of hyperinsulinism the blood sugar response to glucose is diabetic in type in the presence of the steady absorption of depot insulin. The precipitous fall of the blood sugar upon intravenous administration of insulin at the height of the blood sugar curve is again obtained. *However*, prolonged administration of carbohydrate in the presence of continued exogenous hyperinsulinism now fails completely to alter the fixed diabetic, plateau response to a test dose of glucose.

5. The former ability of the animal to convert an insulin-induced diabetic response to a normal one under the influence of high carbohydrate feeding is completely lost when the beta cells have been destroyed and are no longer capable of being stimulated to activity by high carbohydrate feeding.

6. The hypoglycemic phase of the glucose tolerance curve is dependent upon an active and rapid release of insulin into the blood stream in response to the physiologic stimulus for such release. This functional capacity of the beta cells can be depressed by exogenous insulin and awakened by high carbohydrate feeding, and the final response (within the limits of these experiments) is dependent upon the relative intensities of the depressing and awakening stimuli acting simultaneously.

## 22. THE DIFFERENTIATION OF INCIPIENT HYPERTHYROIDISM

ROY E. SWENSON, M.D. (By Invitation), and GEORGE M. CURTIS, M.D.  
COLUMBUS, OHIO

The protein-bound blood iodine is of value in recognizing incipient hyperthyroidism associated with endemic goiter. It is a useful diagnostic tool in differentiating other conditions associated with an elevated basal metabolic rate, particularly when goiter is present.

For the region of Central Ohio, the average normal basal metabolic rate is  $-5 \pm 8$  per cent, while the average normal protein-bound blood iodine concentration is  $0.88 \pm 0.2$  micrograms per 100 milliliters.

When 178 patients with untreated, uncomplicated nontoxic nodular goiter were grouped according to standard deviations from the average normal basal metabolic rate, it was found that the average protein-bound blood iodine progressively increased with each increment of the basal metabolic rate. A significant number of patients had basal metabolic rates of +3 per cent or above and protein-bound blood iodine concentrations of 1.2 micrograms per cent or above.

Of the uncomplicated cases, 66.8 per cent had symptoms suggestive of hyperthyroidism and of these, 70.6 per cent had elevated protein-bound blood iodine concentrations, while only 47 per cent had elevated basal metabolic rates. The protein-bound blood iodine was a better index of thyroid function than the basal metabolic rate alone. Symptoms listed according to their significant correlation to an elevated protein-bound blood iodine are: a pulse of 90 or more per minute, palpitation, weight loss, weakness, nervousness, and emotional instability. Of those with symptoms, 34.5 per cent had elevated protein-bound blood iodide concentrations associated with basal metabolic rates below +3 per cent. Here the incipient hyperthyroidism was unmasked by determination of the protein-bound blood iodine.

Because of the significantly elevated protein-bound blood iodine and the significant incidence of symptoms suggestive of hyperthyroidism in those patients with basal metabolic rates of +3 per cent or above, it is our conclusion that incipient hyperthyroidism is present in those patients with nodular goiter having (1) Basal metabolic rates of plus 4 per cent or above and protein-bound blood iodine concentrations of 1.2 micrograms per cent or above; (2) symptomatology suggestive of hyperthyroidism and a protein-bound blood iodine concentration of 1.2 micrograms per cent or above.

The basal metabolic rate is frequently elevated in association with certain cardiovascular diseases. When goiter is present, the protein-bound blood iodine is a useful diagnostic aid in evaluating thyroid function.

## PROGRAM

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SCIENTIFIC PROGRAM--NOVEMBER 1, 1947

SATURDAY MORNING, 9:15 A.M.

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### 23. INHIBITION OF THE PRESSOR RESPONSE TO EPINEPHRINE INJECTION IN COMPLETE ANOXIA

A. SURTSHIN, M.D. (By INVITATION), S. ROBBARD, M.D. (By INVITATION), AND  
L. N. KATZ, M.D., CHICAGO, ILL.

We have recently observed an unexpected inhibitory effect of complete anoxemia on the pressor response to epinephrine. In the normal dog, an injection of 0.5 mg. of epinephrine intravenously causes a marked rise in arterial pressure which occurs within a few seconds and gradually diminishes during the next few minutes.

In open-chested dogs which are placed on pure nitrogen breathing, the oxygen tension of the blood falls very rapidly from normal to values approaching 0 volumes per cent within ninety seconds. During this period of developing anoxia the blood pressure tends to rise about 40 mm. Hg above the control level. The pressure then begins to fall steadily and this continues until death occurs, unless air breathing is resumed. When air breathing is reinstituted in the control experiments, a sharp postanoxic rise in arterial pressure is observed, this rise being somewhat similar in form to that seen after epinephrine injection in control animals.

The injection of 0.5 mg. epinephrine intravenously during the period of rising blood pressure in developing anoxia results in only a markedly diminished and very transitory rise in arterial pressure. If the anoxia is continued, the pressure falls as in the control anoxia experiments. If the injection is given after the pressure has started to fall in the advanced anoxic stage, epinephrine has no pressor effect and the late anoxic pressure decline continues. Reinstitution of air breathing now results after a brief delay in a typical epinephrine effect with the pressure rising to higher levels than those seen in the control post-anoxia rise.

These results suggest that in conditions of extreme anoxia such as might be seen in drowning or other forms of extreme asphyxia, the injection of epinephrine may lead to no change in arterial pressure inasmuch as the cardiovascular system seems to be incapable of responding to this pressor agent. As a matter of fact, our results show that under these conditions of extreme anoxia, endogenous pressor states exist which, upon the administration of oxygen in adequate amounts, would in themselves cause a marked rise in arterial pressure.

## 24. THE ROLE OF ARTERIAL PRESSURE CHANGES IN THE INDUCTION OF EPINEPHRINE-CYCLOPROPANE IDIOVENTRICULAR RHYTHMS

G. K. MOE, M.D., S. D. MALTON, M.D. (BY INVITATION), WALTER FREYBURGER, B.S. (BY INVITATION), AND BARBARA RENNICK, M.S. (BY INVITATION)  
ANN ARBOR, MICH.

Ventricular extrasystoles, ventricular tachycardia, or ventricular fibrillation can be regularly produced in dogs under cyclopropane anesthesia by doses of epinephrine ranging from 1.0 to 10 micrograms per kilogram. Although the exact mechanism is unknown, it has been observed that dibenzyl-B-chlorethylamine (Dibenamine), which "reverses" the pressor action of epinephrine but does not abolish its chronotropic and inotropic actions, protects the heart against epinephrine-induced idioventricular rhythms. We have been able to demonstrate that Dibenamine prevents ventricular ectopic rhythms *because* it prevents the pressor response to epinephrine.

In dogs, doses of epinephrine which regularly produced ventricular automatism in the control periods failed to do so after the intravenous injection of 15 to 20 mg. per kilogram of Dibenamine. Clamping of the thoracic aorta, which raised arterial pressure by 50 to 100 mm. Hg, also failed to provoke idioventricular discharges in the Dibenaminized animal, but when small doses of epinephrine were followed within ten seconds by aortic occlusion, ventricular tachycardia resulted. After larger doses of epinephrine, which alone failed to arouse ectopic foci, ventricular tachycardia could be induced repeatedly by occlusion, and terminated by release, of the aorta.

In other experiments without Dibenamine, arterial pressure was controlled by means of a pressure stabilizer attached to the abdominal aorta. Doses of epinephrine which regularly established ventricular foci when the stabilizer was clamped off failed to do so when the pressor response was prevented by opening the stabilizer. For example, in one experiment 2 micrograms per kilogram caused a rise of pressure and ventricular tachycardia in control trials with stabilizer closed, but 16 micrograms per kilogram failed to disturb the normal sinus rhythm with the stabilizer open.

Although a rise of arterial pressure greatly facilitates the induction of idioventricular activity by epinephrine, the direct accelerator action of the drug is also important, for elevation of pressure alone will not usually produce more than occasional ventricular extrasystoles associated with reflex vagal slowing of the sinus node.

## 25. AXIS DEVIATION IN HUMAN BUNDLE BRANCH BLOCK

A. MORGAN JONES, M.R.C.P.\* (BY INVITATION), MANCHESTER, ENG., AND  
HAROLD FEIL, M.D., CLEVELAND, OHIO

We have noticed that, in a few cases in which bundle branch block appeared while the patient was under observation, there was little change either in the axis deviation or in the general pattern of the electrocardiogram. This study is an attempt to ascertain how far the axis deviation associated with bundle branch block is determined by the pre-existing electrical axis with normal conduction. From fifty-four patients, in whom both normal conduction and bundle branch block had been recorded, we were able to collect 192 records, ninety showing only bundle branch block, ninety-two showing only normal con-

\*Rockefeller Travelling Fellow.

duction, and ten showing both bundle branch block and normal conduction complexes in all limb leads. In thirty-seven patients adequate chest leads indicated the side of the block, but in the remaining seventeen the side of the block was judged by the limb leads; there were thirty-three patients with left bundle branch block and twenty-one with right. The average position of the electrical axis with normal conduction and with bundle branch block was determined in each case by averaging the axis position in all available records with one type of intraventricular conduction.

In thirty-three patients who developed left bundle branch block, the average shift of the electrical axis associated with the block was 12 degrees to the left; in twenty-one patients who developed right bundle branch block the average shift of axis was 12 degrees to the right. In only twelve patients developing left and ten developing right bundle branch block was the axis shift 15 degrees or more. In the majority of instances the general patterns of the electrocardiogram was closely similar before and after the development of the block. Although there may have been changes in the electrical axis (due to alteration in the position of the heart, ventricular hypertrophy, cardiac infarction, or pulmonary embolism) between the normal conduction and bundle branch block records, these factors could have affected the apparent change of axis in either direction; there is, therefore, no reason to assume that they materially affected the average results, although they must be taken into consideration in individual cases. These extraneous factors can be excluded in the eight cases with normal conduction and bundle branch block complexes in the same record; the results in these cases were similar to those in the whole series.

It is concluded that the axis deviation and the general pattern of the electrocardiogram are not greatly modified by the appearance of bundle branch block and that the axis deviation associated with right and left bundle branch block is due principally to the position of the mean electrical axis of QRS before the bundle branch block appeared.

## 26. REGIONAL PRODUCTION OF MYOCARDIAL NECROSIS WITH ELECTROCARDIOGRAPHIC ANALYSIS

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CHICAGO, ILL.

(INTRODUCED BY G. M. HASS, M.D.)

A new method has been used in producing myocardial lesions, resembling infarcts, in the heart of dogs. The heart is exposed surgically and a hypothermal instrument is applied to a selected area of the epicardium. Lesions, cylindrical in shape, and of controlled dimensions, are then produced by cooling the adjacent myocardium to a low temperature. Lesions of selected dimensions are reproducible in successive animals. Variations encountered in attempts by others to produce lesions by arterial ligation are never observed. Lesions have been repeatedly reproduced in several desired locations in the walls of the auricles and ventricles and in the interventricular septum.

Serial electrocardiographic tracings, using standard limb leads and varied precordial leads, have been obtained at brief intervals. Animals have been sacrificed at various stages in the healing of the lesions up to six weeks, beyond which time all electrocardiographic evidence of cardiac damage has disappeared.

Electrocardiographic analysis indicates that localization of lesions is often inaccurate when standard limb leads and precordial Lead IV are used. These leads are only capable of distinguishing between anterior or apical ventricular lesions and posterior ventricular lesions. Lesions of the right ventricle cannot



be distinguished from those of the left ventricle. The use of standard limb leads and a series of leads from the right and left of the midsternal line has resulted in accurate distinction between lesions of the right and left ventricles, either anteriorly or posteriorly. Evidence of myocardial damage is most pronounced when precordial leads are closest to the lesion. The more distant the leads, the less obvious the changes become. This rule holds only for the first three days. Thereafter, it becomes impossible, even by use of multiple parasternal precordial leads, to distinguish between lesions of the right and left ventricle.

It has not yet been possible to localize large lesions of the auricles or inter-ventricular septum, nor have persistent disturbances in conduction been produced.

## 27. DICUMAROL IN EXPERIMENTAL MYOCARDIAL INFARCTION

L. A. NALEFSKI, M.D. (BY INVITATION), AND GEORGE V. LEROY, M.D.  
CHICAGO, ILL.

In a discussion of possible unfavorable side-effects of dicumarol in the treatment of myocardial infarction, the question was raised whether the hemorrhagic stage of the infarct might be more extensive as a result of the altered clotted activity of the blood. This study was undertaken to investigate the influence of dicumarol on the healing of experimental myocardial infarcts. The anterior descending branch of the left coronary artery was ligated in thirty-two dogs. Seven of these died within twenty-four hours. Dicumarol was administered to sixteen in doses sufficient to maintain the clotting activity of oxalated plasma between 40 and 25 per cent of normal. The remaining nine dogs were controls. The animals in each group were sacrificed from five to twenty-one days after the occlusion of the coronary artery. On gross examination there was no significant difference in the appearance of the infarcts at any stage in the two groups. Mural thrombosis was not found in any heart. Microscopic examination of representative sections did not disclose any obvious difference between the healing infarct in the treated dogs and the controls. Serial electrocardiograms showed no variation in the rate or the character of the evolution of changes associated with anterior infarction in dogs. The sedimentation rate of dogs' blood after coronary occlusion does not follow any consistent pattern, and no influence of dicumarol could be shown by it. Pleural and pericardial adhesions were present to about the same extent in each group of animals.

It is concluded that dicumarol therapy does not have any demonstrable deleterious influence on the healing of experimental myocardial infarction in dogs.

## 28. EXPERIMENTAL PULMONARY INFARCTION: THE ROLE PLAYED BY ABNORMAL PULMONARY CIRCULATION IN THE PRODUCTION OF INFARCTION BY EMBOLUS

DON W. CHAPMAN, M.D., PAUL A. WHEELER, M.D., AND LLOYD GUGLE, M.D.  
HOUSTON, TEXAS

(INTRODUCED BY JAMES A. GREENE, M.D.)

Pulmonary infarction has never been produced consistently in experimental animals. Numerous investigators have found that obstruction of branches of the pulmonary artery is not in itself sufficient to result in infarction, if in-

farection is defined to include necrosis of lung tissue. We have produced pulmonary embolus in twelve normal dogs by the release of venous thrombi and have produced pulmonary congestion in four dogs by the employment of alpha-naphtha-thiourea; pulmonary emboli and congestion were produced by combining these methods in eight dogs.

No infarection resulted in the twelve normal dogs in which intravascular clots were released. Pulmonary congestion was produced in the four dogs receiving alpha-naphtha-thiourea alone, but no gross or microscopic evidence of pulmonary infarection was demonstrable. Of the eight dogs which received alpha-naphtha-thiourea and emboli simultaneously, three developed pulmonary infarection distal to the emboli, and four showed, in addition, hemorrhage distal to the emboli. Apparently, obstruction of a pulmonary artery or one of its branches by an embolus does not cause infarection of the lungs distal to the obstruction because the blood supplied by the bronchial arteries is adequate to maintain nutrition. It appears, therefore, that if the bronchial circulation is interfered with by pulmonary edema or congestion, alveolar necrosis (infarection) results.

## 29. STUDIES ON VASOMOTOR TONE IN THE HYPERTENSIVE STATE\*

S. W. HOOBLER (BY INVITATION), R. H. LYONS, R. B. NELIGH (BY INVITATION),  
H. J. KOWALSKI (BY INVITATION), S. D. MALTON (BY INVITATION),  
W. G. PAINE (BY INVITATION), C. H. MANRY (BY INVITATION),  
J. T. MANNING (BY INVITATION), AND M. M. PEET  
(BY INVITATION), ANN ARBOR, MICH.

Since in arterial hypertension the cardiac output is not usually decreased, it follows that there is an increase in total peripheral resistance. The locus of this increased resistance and its "neurogenic" or "humoral" nature are central problems in a study of the pathogenesis of this disease. The circulatory response to the autonomic blocking agent, tetraethylammonium, can be used in an attempt to quantitate the degree to which vascular resistance is dependent on vasomotor tone in the human subject since we have shown by plethysmographic studies that (1) the drug reduces resistance to blood flow in the limbs to the greatest extent in those areas which can be shown by paravertebral sympathetic block to be under greatest vasomotor tone, (2) reduction of vasomotor tone to a limb by body heating reduces the response to the drug, and (3) after sympathetic denervation of an extremity, peripheral vascular resistance is no longer altered by the drug.

Assuming, therefore, that decreases in vascular resistance after tetraethylammonium are in general proportional to the initial level of vasomotor tone, a comparative study was made of the effects of comparable doses of the drug on the resistance to blood flow in the feet and forearms of twelve hypertensive and twelve normotensive subjects. Resistance was calculated from the formula  $R = \text{mean blood pressure (mm. Hg)}/\text{blood flow (ml./100 ml. limb/minute)}$ . Reductions in resistance were equal in the two groups, thus suggesting that there was no increase in vasomotor tone in the extremities of hypertensive subjects.

The effects of the drug on the renal circulation were also investigated. Here it could be shown that in certain hypertensive subjects renal blood flow

\*Aided by grants from Parke, Davis & Company, The United States Public Health Service, and the Life Insurance Medical Research Fund.

was maintained despite a fall in blood pressure. It would thus appear that increased vasomotor tone may play a significant role in the increased renal resistance of certain hypertensive subjects.

Since blood flow to other vascular areas is less susceptible to accurate measurement, conclusions regarding vasomotor tone from blood pressure observations alone must be accepted with some reservations since not only changes in peripheral resistance but also changes in pulse rate, arterial elasticity, and cardiac output may play a role in the depressor response to the drug. Two lines of evidence would indicate, however, that in other vascular areas than the extremities increased vasoconstrictor tone may be present in the hypertensive subject: (1) After exclusion of both lower legs and one upper extremity from the circulation by arterial tourniquets, the injection of tetraethylammonium still produces a blood pressure fall in hypertensive subjects but rarely in normotensive subjects; (2) after supradiaphragmatic splanchnicectomy the average response of the blood pressure to an injection of the drug is materially decreased.

These studies would seem to provide evidence that in some hypertensive subjects there is an increase in neurogenic vasomotor tone to the splanchnic or renal vascular beds but not to the blood vessels of the extremities.

### 30. THE EFFECT OF TETRAETHYLAMMONIUM CHLORIDE ON THE BLOOD FLOW IN THE EXTREMITIES OF NORMAL INDIVIDUALS

OWEN L. SLAUGHTER, M.D., HUGH S. BROWN, M.D., AND KHALIL G. WAKIM, M.D., ROCHESTER, MINN.

The effect of tetraethylammonium chloride on the blood flow in the extremities of seven normal individuals (four male and three female) was studied by the use of an air displacement plethysmograph with a compensating spirometer recorder. Tetraethylammonium chloride was administered to each individual intravenously in amounts of 300 to 400 mg. at the rate of 100 mg. per minute. All experiments were conducted in room temperatures which ranged from 80 to 85° F. (26.6 to 29.4° C.), but no single experiment was done in which there was a variation of more than 1° F. (1.8° C.) in room temperature.

With the subjects lying on the test bed, the plethysmographs were applied to the forearms and legs. The arm plethysmograph included the hand and forearm up to one inch above the olecranon process, and the leg plethysmograph included the foot and leg up to one inch below the tibial tuberosity. After the subject was well adjusted to the surroundings, determinations of blood flow were made for a period of about thirty minutes in order to establish a control level. The drug was then injected intravenously and records of the blood flow were taken for thirty to forty-five minutes thereafter.

The subjective symptoms, after administration of the drug, were a metallic taste, numbness and tingling, tachycardia, dryness of the mouth, and variable disturbances of vision with impairment of accommodation.

The average blood flow in the forearms of all seven subjects before the administration of tetraethylammonium chloride was 4.3 ml. per 100 ml. of limb volume per minute (range, from 1.7 to 7.3 ml.). After the injection of tetraethylammonium chloride the blood flow increased in every case, with a maximum increase occurring between five and fifteen minutes after injection (average about nine minutes). In all seven cases the average maximal blood flow in the forearms, after the injection of tetraethylammonium chloride, was 10.1 ml. per 100 ml. of forearm volume per minute (range, from 3.4 to 14.7 ml.). This rep-

resents an increase of 87 per cent in the average over the control flow. The average control blood flow in the legs of all seven subjects before administration of tetraethylammonium chloride was 3.2 ml. per 100 ml. of limb volume per minute (range, from 1.7 to 4.5 ml.). After injection of the drug the blood flow in the legs also increased in every case and reached a maximum at the same time as in the arms. The average maximal blood flow in the legs, after injection of tetraethylammonium chloride, was 6.9 ml. per 100 ml. of leg volume per minute (range, from 4.0 to 9.5 ml.). This represents an increase of 116 per cent over the control value (range, from 60 to 200 per cent). The increased blood flow in both upper and lower extremities gradually regressed; nevertheless, at the termination of the experiment (thirty to forty-five minutes after the administration of tetraethylammonium chloride), the flow still was higher than the control value. Accompanying the increase in blood flow there was a rise in cutaneous temperature.

### 31. THE NATURE OF THE COLD PRESSOR TEST AND ITS SIGNIFICANCE IN RELATION TO NEUROGENIC AND HUMORAL MECHANISMS IN HYPERTENSION

MORTON, F. REISER, M.D. (BY INVITATION), AND EUGENE B. FERRIS, JR., M.D.  
CINCINNATI, OHIO

Previous evidence suggests that both neurogenic (autonomic) and humoral factors are concerned in the pathogenesis of increased arteriolar tone in clinical hypertension, but the exact nature of such mechanisms and their relative importance and interrelationships are not well understood. The present study is concerned with the effector mechanism of the cold pressor response in hypertension, utilizing varying degrees of sympathetic block induced by spinal anesthesia and tetraethylammonium chloride. The results clarify the effector mechanism of the cold pressor test and indicate that its utilization along with other procedures may be helpful in evaluating the relative importance of neurogenic and humoral components in patients with this disease.

In each of twenty hypertensive patients the cold pressor response was elicited in the usual manner and then repeated after the administration of tetraethylammonium chloride. One additional patient was tested in a similar manner four months after partial sympathectomy. In eight cases the response was again tested after high spinal anesthesia and in five of these repeated as the anesthesia receded to lower levels. In five instances the effect of tetraethylammonium chloride, was again tested following the performance of the test under spinal anesthesia.

Two phases of the blood pressure response to cold were noted: (1) the response during the minute of exposure to cold (cold minute), the classical cold pressor response, and (2) delayed pressor responses after the hand had been removed from cold water. In all cases tetraethylammonium chloride abolished the cold minute response, demonstrating that the effector phase of this reflex is neurogenic in character and indicating that the cold pressor response may be useful in evaluating procedures designed to eliminate autonomic tone in hypertension. Release of varying portions of the arteriolar bed from active vasomotor control (by spinal anesthesia) produced dampening of the response which tended to vary directly with the extent of denervation, but the smallest portion of the arteriolar bed which, under active vasomotor control, was capable of exciting clinically significant cold pressor reactivity varied from patient to patient.

In one case, who showed marked anxiety and resentment in response to the cold pressor test, there occurred following the cold minute exposure (during tetraethylammonium chloride action) a very sharp transient rise in pressure. Similar, but less marked, delayed transient elevations were seen in eleven other patients. Since such a pressor response occurs at a time when autonomic impulses are blocked at the ganglia, it must be the result of a humoral agent (the action of such agents not being eliminated by tetraethylammonium chloride).

Six (30 per cent) of the twenty patients failed to obtain a depressor effect from the administration of tetraethylammonium chloride, yet the cold pressor response was eliminated in each, suggesting that circulation of a humoral pressor agent, rather than failure of the drug to produce satisfactory autonomic blockade, was responsible for the absence of depressor response in these patients.

The data suggest that both humoral and neurogenic (autonomic) mechanisms may interact as clinically important factors in hypertension.

## 32. CIRCULATORY RESPONSES TO SPINAL AND CAUDAL ANESTHESIA IN HYPERTENSION: RELATION TO THE EFFECT OF SYMPATHECTOMY\*

### II. EFFECT OF RENAL FUNCTION

A. C. CORCORAN, M.D., ROBERT D. TAYLOR, M.D., AND IRVINE H. PAGE, M.D.  
CLEVELAND, OHIO

In 1944 we found that the renal effects of high spinal anesthesia in patients with essential hypertension were variable. We suggested that the type of response of the renal vasculature might mirror the nature of the hypertensive process. Renal vasodilatation might then be taken as evidence of pre-existing increased vasomotor tone and failure of such vasodilatation as evidence of humoral vasoconstriction or of vascular rigidity due to renal arteriosclerosis. It was intimated that those patients who had predominance of vasomotor influence might receive more benefit from sympathectomy than would others.

The present report includes observations on the renal functional effects of high spinal anesthesia administered to seventeen patients with essential hypertension and of caudal anesthesia given to eighteen patients. Fourteen of the latter group had lumbodorsal sympathectomy.

The effects of spinal and caudal anesthesia upon renal hemodynamics were similar. Reduction of the average arterial pressure of hypertensive patients by 20 mm. Hg, to levels ranging from 85 to 125 mm. Hg, usually causes renal vasodilation, resulting in increased renal blood flow and a slight decrease in glomerular filtration rate.

Qualitatively the renal vascular response of normotensive and hypertensive patients to these procedures is identical. Quantitatively, they may differ slightly in that in hypertensive patients a fraction of afferent arteriolar resistance is not removed by anesthesia.

The renal vasodilator response to anesthesia in hypertension indicates that a large proportion of increased renal vascular resistance in this disease is dependent on nervous influences which are affected by anesthesia extending to about D 5, or alternatively that denervation by anesthesia has sensitized the vessels to vasodilator influences.

The major hemodynamic change induced by spinal or caudal anesthesia is seen to be due to decreased peripheral resistance.

\*From the Research Division of the Cleveland Clinic Foundation.

Exceptionally, a few hypertensive patients show deficient or slowed renal vasodilator responses to spinal or caudal anesthesia. Two such patients failed to benefit from lumbodorsal sympathetic ganglionectomy, but operation was equally ineffective in patients whose responses were vasodilator. The renal vascular response to anesthesia is not a positive guide in the selection of patients, although the absence of vasodilation during anesthesia may contraindicate operation.

### 33. RELIEF OF CAUSALGIA BY USE OF TETRAETHYLAMMONIUM CHLORIDE

JAMES E. CONLEY, M.D. (BY INVITATION), HERMAN C. SCHUMM, M.D. (BY INVITATION), FRANCIS F. ROSENBAUM, M.D., AND FREDERICK G. GAENSLER, M.D. (BY INVITATION), MILWAUKEE, WIS.

Tetraethylammonium chloride was used for sympathetic blockade to relieve pain in seventeen patients with causalgia. The patients were selected on the basis of complaint of pain without consideration of trophic or vasomotor disturbances. In twelve patients pain was severe enough to prevent proper physiotherapy. Tetraethylammonium chloride was used primarily to obtain symptomatic relief.

Five patients with Sudeck's primary bone atrophy were given this drug in doses of 200 to 500 milligrams. This was sufficient to maintain comfort and to permit physiotherapy. Relief from pain appeared promptly and lasted several hours to forty-eight hours, even though complete sympathetic blockade was not produced. Follow-up clinical and radiologic studies showed improvement in the bone atrophy.

Two patients who were bedridden because of acute osteoporosis of the spine received relief from tetraethylammonium chloride which was comparable to that afforded by the use of narcotics. One of these patients was given 500 mg. to 2.0 Gm. in divided doses daily. She became ambulatory immediately with the additional aid of an orthopedic corset.

Three patients with bilateral thrombophlebitis whose walking was limited by cramps in the legs received extensive therapy with this drug. The relief from pain was not striking, although large doses were beneficial for short periods. The therapy did seem to help somewhat in restoring these patients to full activity.

Tetraethylammonium chloride was given to five patients with causalgia in the presence of known orthopedic disorders. These patients were selected because their pain seemed unrelated and disproportionate to the structural lesion. The bizarre pain was relieved, but the legitimate pain persisted. In two of these patients, swelling of the involved extremities decreased after tetraethylammonium chloride was given.

One patient with multiple pelvic fractures with bilateral pain in the legs had prompt relief of pain lasting about six hours after 225 mg. of the drug. This remission of pain permitted her to move the legs for the first time in nine weeks. Narcotics were required much less frequently thereafter.

A young woman with advanced scleroderma who had pain on motion of the fingers was able to use her hands with much less discomfort after moderate doses of the drug, but there was no significant change in the underlying disorder.

Tetraethylammonium chloride is of definite value in relief of causalgia. The relief of pain may be striking even though the sympathetic blockade is not complete enough to produce increased warmth and dryness of the extremities. The relief is usually sufficient to permit appropriate physiotherapy.

### 34. THE EFFECT OF DITHIOPROPANOL (BAL) ON HUMAN LEAD INTOXICATION

HENRY W. RYDER, M.D., AND ROBERT A. KEHOE, M.D., CINCINNATI, OHIO

Evidence has been accumulating in support of the hypothesis that certain heavy metals are toxic to biologic systems because of their reversible combination with the sulfhydryl groups of the protein fraction of cellular enzymes. There is some evidence that lead may be included in the group of metals that behave in this manner. Barron and Kalnitsky have shown that dithiopropanol (BAL) is one sulfhydryl-containing compound which is capable of reversing such an enzymatic inactivation due to lead. On the other hand, Braun, Lusky, and Calvery felt that lead and dithiopropanol exerted additive toxic effects in lead poisoning as induced experimentally in rabbits.

We have studied thirty men with acute episodes of lead poisoning, ten of whom were treated with dithiopropanol to the limit of tolerance. The drug has no dramatic immediate effect on the symptoms of lead poisoning. As a group the treated men had no different clinical course than the group of untreated men, except for the characteristic local and general effects of dithiopropanol and the occasional production, after prolonged therapy, of generalized muscular aching.

In contrast to the absence of clinical effect, the drug had unprecedented metabolic effects. Characteristically, the cellular lead concentration dropped sharply within a few minutes after the administration of BAL, continued to fall for an hour or longer, and returned to the previous level within twenty-four hours. The urinary lead concentration increased from several to fiftyfold within an hour or so. On the other hand, the lead concentration in the blood plasma of all the men was of a uniform order of magnitude, whether or not they had had abnormal lead exposure, clinical lead poisoning, or dithiopropanol therapy. The treatment did not change the fecal lead concentration or output.

While the changes in the lead concentration in the erythrocyte and urine were obtained again and again following repetitive administration of the agent, they were transient, and not enough lead was removed by the therapy to increase materially the total amount of lead eliminated by the subjects during the period of intermittent therapy or in the weeks that followed.

In summary we have observed that dithiopropanol is of no value in the treatment of acute poisoning in adults due to inorganic lead. We have observed also that the drug, in contrast to the lack of clinical effect on the symptoms of poisoning, causes a transient decrease in the erythrocyte lead concentration and a temporary increase in the urine lead concentration to a degree we have not seen with other types of therapy.

From these two observations we infer that the symptoms and signs of lead poisoning and the storage and elimination of lead may be due to mechanisms that are not intimately related. Our clinical experience in the lead trades supports this inference. The observed effects on lead metabolism are consistent with the hypothesis that lead combines with an erythrocyte component and that this combination can be reversed in vivo by dithiopropanol therapy, but the failure of symptomatic response to therapy does not give support to the theory that this particular enzyme system is involved in the mechanism of lead poisoning.

## TO BE READ BY TITLE

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### 35. CONGENITAL FAMILIAL HYPOPROTHROMBINEMIAS

ARMAND J. QUICK, M.D., MILWAUKEE, WIS.

The experimental evidence of the writer that prothrombin is a complex composed of several components suggests the possibility that more than one type of hypoprothrombinemia may occur clinically. This was borne out by studies of two families, in each of which several members have a hypoprothrombinemia. In the first family the mother, one son, and one daughter have a prothrombin time of  $15\frac{1}{2}$  to 16 seconds. On mixing their plasmas with an equal volume of normal plasma, a mixture is obtained which has a prothrombin time of  $13\frac{1}{2}$  seconds, the expected calculated value based on the writer's prothrombin curve. This type of hypoprothrombinemia is similar or identical with that produced by dicumarol poisoning. In this type of hypoprothrombinemia, it is component B which is reduced.

In the second family, two brothers are affected. Each has a prothrombin time of 19 to 20 seconds. Both have a bleeding tendency. When their plasmas are mixed with an equal volume of normal plasma, a normal prothrombin time (12 seconds) characteristically results. This same behavior has been noted with plasma from patients with avitaminosis K. When plasma from a member of family I is mixed with an equal volume of plasma from a member of family II, a prothrombin time of  $13\frac{1}{2}$  seconds results, thus showing that the second plasma has the same corrective capacity on the first as normal plasma. In other words, the first plasma contains an excess of the factor lacking in the second plasma (component A), whereas the second plasma contains as much component B as normal plasma.

The prolonged prothrombin time resulting when oxalated plasma is stored is due to the disappearance of a principle that is neither component A nor B. It is not adsorbed by  $\text{Al}(\text{OH})_3$ ,  $\text{Ca}_3(\text{PO}_4)_2$  and similar agents. This substance has been tentatively named the labile factor. Its lack in plasma may apparently cause a hemorrhagic diathesis as the recent report of Owren suggests.

The prothrombin time serves as a reliable guide of hemostatic efficiency even though it is dependent upon three factors: components A and B and the labile principle. A hemorrhagic tendency appears when the prothrombin time is 19 seconds or longer.

### 36. THE BLOOD COAGULATION-RETRACTION TIME AND ITS RELATION TO THROMBOSIS\*

JOHN S. HIRSCHBOECK, M.D., MILWAUKEE, WIS.

Clot retraction is dependent upon thrombocytes, surface forces, erythrocyte mass, and qualitative and quantitative variations in fibrin. The same factors are also important elements in the development of the thrombotic tendency in individuals.

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\*Supported by a Grant-in-aid from the United States Public Health Service.



A method which measures the combined coagulation and clot retraction time of capillary blood was used in the study of 100 normal individuals, 100 post-operative patients, and 300 hospitalized medical patients. The coagulation-retraction time is determined by suspending a 20 c.mm. drop of capillary blood in castor oil. The drop is observed at five-minute intervals for the development of a bud of serum which projects into the oil from the retracting clot. The normal coagulation-retraction time is longer than 20 minutes, the average for 100 individuals being 33.1 minutes.

Patients in the postoperative period have shortened coagulation-retraction times. Approximately one-third of these patients had coagulation-retraction times less than 20 minutes during most of the postoperative period. Of the 300 general medical patients, 41 per cent had coagulation-retraction times less than 20 minutes and 11 per cent less than 15 minutes. One patient in this group developed pulmonary embolism when her coagulation-retraction time was 11 minutes (the shortest time observed in over 3,000 determinations).

The coagulation-retraction test is well adapted to the control of heparin therapy and can be used instead of the ordinary coagulation time determinations. The test is not reliable in following dicumarol therapy because its correlation with prothrombin time is not accurate. It is suggested that patients whose coagulation-retraction time is consistently less than 20 minutes be given prophylactic anticoagulant therapy.

### 37. THE EFFECT OF FOLIC ACID ON RADIATION INDUCED ANEMIA AND LEUCOPENIA

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ERIC SIMMONS, PH.D. (BY INVITATION), CHICAGO, ILL.

Penetrating radiations originating external to the body and certain radioactive elements deposited within the body produce anemia, leucopenia, and other effects on the blood and blood-forming tissues when given in adequate doses. It is of interest that certain species develop a striking increase in mean corpuscular volume concomitant with the anemia after exposure to these physical agents. Experiments have been conducted on the human, rabbit, and rat in which attempts were made to modify these hematologic effects of radiations by the enteral and parenteral administration of folic acid. The penetrating radiation used in these studies was confined to x-ray (200 kv.) and was administered to the whole body in single (rabbits) or divided doses (human beings). The radioactive elements used were  $P^{32}$  (human beings) and  $Sr^{90}$  (rats) given intravenously in the former and intraperitoneally in the latter subjects. In certain of these experiments the folic acid was given before the radiation or radioelement was administered and continued. In others the folic acid was begun approximately at the point of maximum anemia or leucopenia or both and continued.

The anemias and leucopenias induced by these radiations were not prevented nor was recovery hastened by the administration of folic acid in adequate doses. The macrocytic anemia produced in rats by radiostrontium and in human beings by total body x-radiation or radiophosphorus was likewise unaffected.

### 38. COMBINED SYSTEM DISEASE AND HEMATOLOGIC RELAPSE OCCURRING IN PERSONS WITH PERNICIOUS ANEMIA TREATED WITH SYNTHETIC FOLIC ACID (PTEROYL GLUTAMIC ACID) FOR A PERIOD OF TWO YEARS

RICHARD W. VILTER, M.D., CARL F. VILTER, M.D. (BY INVITATION), AND VIRGINIA HAWKINS, R.N. (BY INVITATION), CINCINNATI, OHIO

The observations presented have evolved from an extension and elaboration of studies undertaken in November, 1945, and reported at this meeting one year ago. Elucidation of the following questions has been sought: (1) Will synthetic folic acid given orally or parenterally control the anemia and glossitis of pernicious anemia and sprue for long periods of time as successfully as liver extract? (2) Will synthetic folic acid therapy prevent the development of the neurologic manifestations of pernicious anemia or control them after they have appeared?

Twenty-eight persons with pernicious anemia, the majority of whom had been controlled for two to seventeen years by injections of liver extract, were treated with folic acid orally for six months to two years. Folic acid treatment of twenty-four of these was begun on or about November, 1945; the remaining four have been treated six to eighteen months. Oral doses ranged from 30 mg. three times a week to 50 mg. daily.

Nine patients with pernicious anemia, six of whom were in relapse and three of whom previously had been maintained on parenteral liver extract, were treated with folic acid parenterally for six to twelve months. Doses ranged from 30 mg. daily to 30 mg. weekly. Three persons with sprue and one with macrocytic anemia secondary to ileosigmoidostomy were observed for nineteen to twenty-five months while receiving varying doses of folic acid orally. Complete physical and neurological examinations, erythrocyte counts, hemoglobin levels, hematocrit determination, and reticuloocyte counts were performed at the beginning of the study and were repeated as often as the condition of the patient demanded.

So far, combined system disease has occurred in eight of the thirty-seven persons with pernicious anemia. During the first year four of the patients given folic acid orally developed posterolateral sclerosis after five to eight months of therapy. One of these showed mental deterioration. In the second year three patients on oral and one on parenteral therapy developed signs of posterolateral sclerosis, two with distinct mental changes, within four to eighteen months of the beginning of treatment. Satisfactory remissions followed the administration of liver extract intramuscularly.

Hematologic values have neither reached nor been maintained at optimum levels in the nine persons treated parenterally and one-third of the persons receiving folic acid orally developed moderate degrees of anemia. Gastric carcinoma and unexplained rectal bleeding accounted for the anemia in two of these. One patient had a striking hematologic and neurologic relapse after eighteen months of oral therapy (30 mg. three times a week). After intramuscular folic acid therapy for two weeks (30 mg. three times a week) the relapse continued unchecked. Erythrocytes had fallen to 1.51 millions and hemoglobin to 7.2 grams. Urine assays indicated that 50 to 80 per cent of the folic acid administered was excreted in the urine within twenty-four hours. The patient was given 12 Gm. of 5-methyl uracil daily for ten days. Reticuloocytes rose to 10 per cent on the fourth day of this treatment and there was a

subsequent rise in red cells and hemoglobin to 2.45 millions and 9.5 grams. After discontinuance of 5-methyl uracil, complete hematologic and partial neurologic remission was achieved by parenteral liver therapy. Distinct glossitis occurred in five of the patients with pernicious anemia during folic acid therapy and waxed and waned without relation to the administration of the drug. The persons with sprue and secondary macrocytic anemia were maintained in better clinical and hematologic states than had resulted from liver extract therapy.

In conclusion, the second year of this study demonstrates that folic acid will not maintain hematologic remissions in all patients with pernicious anemia nor will it prevent the appearance of glossitis. In addition it confirms the previous observations that posterolateral column disease and mental deterioration may develop in persons with pernicious anemia even though their hematologic state is well controlled by folic acid.

### 39. TREATMENT OF PERNICIOUS ANEMIA WITH PTEROYL GLUTAMIC ACID (FOLIC ACID).

STEVEN O. SCHWARTZ, M.D., AND BERTHE E. ARMSTRONG, M.D., (BY  
INVITATION), CHICAGO, ILL.

The paper is a résumé of experiences with folic acid in the treatment of pernicious anemia. The studies were begun in January, 1946, and over 100 patients have been maintained for varying periods of time on 5 mg. doses (daily). The discussion will center on such patients as have been either taken off the drug or maintained for twelve months or longer. In this category are sixty-nine patients. Of these, thirty-eight were men and thirty-one were women. The ages varied from 29 to 80 years. Six were placed on folic acid therapy immediately after an "assay period," while sixty-three were changed from routine maintenance liver therapy. Fifty-two are still on folic acid (as of September, 1947), four have disappeared from observation, and thirteen have been taken off the drug for the following reasons:

1. Hematologic relapse—five patients—during the sixth, seventh, eighth, twelfth, and thirteenth months.
2. Neurological relapse—four patients—during the second, sixth, eighth, and twelfth months.
3. Progressive anemia (not real relapse)—one patient—during the thirteenth month.
4. Refusal to take folic acid—one patient—because of "headaches."
5. Unreliability in taking folic acid—one patient.
6. Unreliability of subjective complaints—one patient.

It is concluded from the studies so far performed that 5 mg. folic acid per day is inadequate to prevent clinical, neurological, or hematologic relapses. Studies are now in progress evaluating the efficacy of 10 mg. per day.

## 40. EXOGENOUS HEMOCHROMATOSIS

STEVEN O. SCHWARTZ, M.D., AND SUNOLL BLUMENTHAL, M.D.

(BY INVITATION), CHICAGO, ILL.

Five female patients, with severe anemias due to miscellaneous causes, who received multiple transfusions and developed hemosiderosis and fibrosis of the liver were studied. The patients ranged in age from 19 to 75 years and were followed from nine months to four and one-half years. They received from twenty-one to seventy-five blood transfusions. With the evidence available from the study of the cases, together with a review of eight similar previously reported cases in the literature, the conclusion is reached that hemochromatosis in man can be experimentally produced by the administration of large amounts of iron (in the form of hemoglobin) if the iron is not secondarily lost from the body.

That the cases were not primary hemochromatosis is evidenced by the fact that the disease occurred in women, many of whom were young, and was accompanied by a severe anemia.

The pathogenesis of both idiopathic (endogenous) hemochromatosis and secondary (exogenous) hemochromatosis is discussed.

With the increased use of blood transfusions and the consequent lengthening of the anemic patient's life, the incidence of exogenous hemochromatosis will increase and will afford an opportunity to study a hitherto poorly understood disease entity.

## 41. MYELOMA CELL CYTOLOGY AS REVEALED BY HISTOCHEMICAL METHODS\*

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(INTRODUCED BY LOUIS R. LIMARZI, M.D.)

There has been no attempt made thus far to relate the cytoplasmic vacuoles, areas of hyaloplasm, and perinuclear clear area of the myeloma cells, as revealed by the Romanowsky stains, to the presence of preformed cytoplasmic organoids. In five cases of multiple myeloma of the "plasma cell" type, sternal marrow and air-dried marrow smears were studied by various cytologic and cytochemical techniques. By these means it has been possible to correlate cytoplasmic structures (mitochondria and portions of the Golgi element) with these cytoplasmic clear areas and, in part, to determine their chemical composition.

Marrow smears stained with Wright's and May-Grünwald-Giemsa stains indicated that these cytoplasmic clear areas (variously described by other authors) exhibited a series of distinctive patterns. Rodlike, filamentous, circular, and dotlike areas of hyaloplasm could be readily delineated. These light areas were found to be either diffusely scattered throughout the cytoplasm or congregated on one side of the nucleus, giving rise to a "Hof" or perinuclear clear zone.

Supravital studies on aspirated sternal marrow, utilizing neutral red and the neutral red-Janus green combination, demonstrated a variety of mitochondria whose form and distribution were identical with the clear areas noted in the stained smears. Neutral red vacuoles were present in all of the myeloma cells; however, they appeared to contribute but a small share to the total cytoplasmic pattern.

\*Work done while Research Fellow, Department of Medicine, University of Buffalo School of Medicine, Buffalo, N. Y.

The Bensley-Cowdry mitochondrial technique revealed the presence of numerous mitochondria whose general configuration and distribution within the cells correlated with the observations made on supravital study.

The myeloma cells, when stained with the fat stains sudan black B and sudan IV, exhibited numerous sudanophilic organoids which were identical cytologically with the structures demonstrated by the aforementioned techniques. These bodies were not stained by sudan III, nor did they exhibit a positive Schultz reaction. They were stained by Nile blue sulfate; however, the staining was of the nonspecific type. In view of these negative findings, neutral fats, cholesterol, and cholesterol esters were excluded as being responsible for their sudanophilic properties. The organoids were stained by the Smith-Dietrich method, indicating their phospholipid nature.

The cytochemical test for vitamin A and/or its carotene precursors was positive and was precisely localized to the cytoplasmic organoids under discussion. Thus far it has been impossible to localize vitamin C within this group of cells.

All attempts at the cytochemical localization of the aromatic group of amino acids and the sulfhydryl-containing amino acids within these intracellular structures have been uniformly unsuccessful. A positive reaction for the free alpha amino acid groups has, however, been obtained.

Phase microscopy was used in this study as a method of providing a positive correlation between the mitochondria and neutral red vacuoles and the clear areas in the basophilic spongiosum.

It is concluded that (1) the areas of hyaloplasm in the myeloma cells are due to the presence of underlying mitochondria and portions of the Golgi element; (2) these structures have been found to be of a phospholipid nature; (3) vitamin A and/or the carotinoid pigments are localized to these areas; (4) these structures give a cytochemical reaction for free alpha amino acid groups.

## 42. BLOOD AND BONE MARROW FINDINGS IN RENAL DISEASE

IRWIN R. CALLEN, M.D. (BY INVITATION), LOUIS R. LIMARZI, M.D., CHICAGO, ILL.  
AND HAROLD C. LUETH, M.D., OMAHA, NEB.

The association of anemia and renal impairment has been recognized for some time. Because the anemia is refractory to treatment and has no positive distinguishing characteristics, it has not been diagnosed in many instances. In addition there has been no agreement as to the hematologic findings.

In this study 102 patients were included, divided into 44 with chronic glomerulonephritis with azotemia, 22 without azotemia, 20 with essential hypertension, 6 with acute glomerulonephritis, 8 with miscellaneous kidney diseases, and 2 with extrarenal uremia. As a result of these studies it has been shown that anemia as demonstrated by the hemoglobin, red blood count, and hematocrit values, in general, is inversely related to the elevation of the nitrogenous waste products in the peripheral blood. The hemoglobin averaged 8.5 Gm. per 100 ml., the red blood count, 2,900,000 per cubic millimeter, and the hematocrit, 25 per cent, in cases of chronic glomerulonephritis, with an average nonprotein nitrogen of 123 mg. per 100 milliliters. The leucocyte count averaged about 12,500 in the cases of acute glomerulonephritis. Chronic types of renal disease showed borderline elevations of the leucocyte count. A slight increase in the relative percentage of stab neutrophils was also present in these respective cases. The average of the mean corpuscular volume, hemoglobin, and hemoglobin concentration in these studies proved to be within normal

limits for these groups. There were individual variations, but generally they involved the hemoglobin values and not those of the mean corpuscular volume as had been reported by other investigators.

A further correlation of the retained nitrogenous products and the values for the hemoglobin, hematocrit, and red blood count showed that in ten of the twenty-two patients with chronic glomerulonephritis without azotemia a significant variation was noted. Eight of these ten revealed a rise in one or two of the four blood chemistry determinations (nonprotein nitrogen, urea-nitrogen, uric acid, and creatinine).

The bone marrow, in contrast to some of the other published data, revealed a fairly adequate cellular picture with a myeloid-erythroid ratio near the normal in all the groups. The patients with acute and chronic glomerulonephritis with azotemia showed a slight increase in myeloid elements. The relative percentage of polychromatic normoblasts, however, was normal in all groups. The myeloid-erythroid layer, determined following centrifugation of 1 ml. of marrow blood in a hematocrit tube, was not increased in any of the groups except in acute glomerulonephritis. This increase was due to an increase in myeloid elements.

As a result of these studies, the following conclusions can be drawn: (1) anemia in renal disease is inversely related to the levels of nitrogenous waste products in the blood; (2) in cases where the anemia is borderline and renal disease is suspected, not only one, but complete blood chemistry studies should be done serially; (3) the bone marrow is relatively normal with some evidence of myeloid hyperplasia. Hypoplasia or maturation arrest of the bone marrow elements was not observed.

#### 43. A STUDY OF THE MARROW IN ARTHRITIS

EUGENE F. TRAUT, M.D., AND STEVEN O. SCHWARTZ, M.D., CHICAGO, ILL.

Since evidence regarding the nature of arthritis has been sought often in the peripheral blood, it was thought advisable to study the most important blood-building organ, the marrow. We were interested in learning whether the marrow would help distinguish the two great groups of nonspecific chronic arthritis and whether the marrow findings paralleled the clinical progress in these conditions.

Forty arthritic patients were studied: nineteen atrophic, fifteen hypertrophic, two psoriatic, and four patients with rheumatic fever. These patients were in various stages of activity. Two of the patients with atrophic arthritis had 2 marrow studies.

One of us (S. O. S.) studied the marrows and gave a report without access to other clinical findings.

In general, the marrow mirrored the clinical impression gained otherwise. The marrow study gave further evidence of the inflammatory nature of atrophic (rheumatoid) arthritis as compared with hypertrophic arthritis. Complicating infections can cause the myelogram of degenerative joint disease to resemble that of inflammatory arthritis. The changes in the marrow reflect the course of disease in the joints.

The findings in the few patients with rheumatic fever suggest marrow studies as a measure of the activity of the rheumatic process.

#### 44. RESULT OF METHYL-BIS (BETA-CHLOROETHYL) AMINE HYDROCHLORIDE THERAPY: A PRELIMINARY REPORT OF A NITROGEN MUSTARD

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(INTRODUCED BY JAMES A. GREENE, M.D.)

Fourteen patients with confirmed diagnosis of lymphoma, leucemia, and multiple myeloma received single courses of methyl-bis (beta-chloroethyl) amine hydrochloride intravenously in the usual dosage.

Of six patients with Hodgkin's disease, two had an excellent remission and one a satisfactory response, whereas the remaining three improved either minimally or not at all. Nitrogen mustard administration provoked only partial remissions in two cases of lymphosarcoma and minimal improvement in two cases of chronic myelogenous leucemia. One patient with chronic lymphogenous leucemia obtained a rapid decrease in the leucocytosis without other evidence of benefit.

Two patients with severe skeletal pain due to multiple myeloma were improved symptomatically to a striking degree, but roentgen studies failed to demonstrate regression of the bone lesions. A case of mycosis fungoides was unaffected by nitrogen mustard therapy.

Nine patients of the entire series had received no previous treatment, whereas five had been subjected to varying amounts of roentgen therapy. In this limited clinical experience the previous use of irradiation did not appear to affect the response to the nitrogen mustard in an adverse way.

Excluding the three cases of leucemia, a significant leucopenia followed the administration of the drug in five patients (42 per cent of the series), but no ill effects ascribable to the leucopenia were detected. Nausea and vomiting occurred in 64 per cent of these patients during the course of treatment and constituted the only clinical evidence of toxicity in this series.

#### 45. EXPERIENCES WITH URETHANE IN EIGHT PATIENTS WITH MALIGNANT DISEASE

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(BY INVITATION), E. A. SHARP, M.D., AND E. C. VONDER HEIDE, M.D.

DETROIT, MICH.

The therapeutic action and toxic effects of urethane have been studied in eight patients with leucemia and other neoplastic conditions. Oral administration was tolerated by most patients in doses of 0.5 to 6.0 Gm. daily over periods ranging from nine to fifty-four days. Total doses ranged from 26.5 to 221 grams.

No changes were noted in renal or liver function as observed by complete urinalysis, phenolsulphthalein, urea clearance, bromsulphthalein, hippuric acid, and cephalin flocculation tests.

There was little or no clinical improvement in three of four patients with leucemia exhibiting hematologic responsiveness and decrease in size of enlarged lymph nodes, spleen, or liver. Urethane did not affect the progress of terminal subacute leucemia, myelomatosis, or carcinoma of the lung. Although nausea and vomiting occurred frequently, there was no evidence that these symptoms constitute an indication for stopping urethane treatment. Weight loss out of

proportion to reduced food intake was noted in four patients and may represent an important constitutional injury to patients receiving urethane. Transient drowsiness and slight dizziness were observed.

Urethane caused a fall in the absolute numbers of both lymphoid and myeloid leucocytes in the blood of patients with normal or leucemic counts. The effect is greater on the more undifferentiated leucocytes. Severe temporary hypoplasia of all elements of the bone marrow may result from overtreatment and may progress after treatment has been stopped. Megakaryocytopoiesis persists longer than erythrocytopoiesis or leucopoiesis and appears to recover earliest after urethane therapy.

In the chronic leucemias, initial counts between 22,350 and 133,800 per cubic millimeter dropped to levels ranging between 1,900 and 12,000 per cubic millimeter after the patients received from 70 to 116 Gm. of urethane. One patient with chronic myelogenous leucemia exhibited a rise in the platelet count although the leucocyte count dropped to normal. There is no correlation between daily or total dose of urethane and the rate or extent of change of the total leucocyte count, lymph nodes, liver, spleen, or bone marrow.

No significant changes in reticulocyte percentages or color indices were observed. Anemia was favorably influenced in some patients although in others it became more severe.

There was regression of lesions in cases of mycosis fungoides and carcinoma of the skin.

On the basis of our studies it appears necessary to make bone marrow examinations, determinations of absolute leucocyte counts, and estimation of platelets, hemoglobin, and erythrocyte levels frequently.

#### 46. BLOOD COAGULATION DURING INTRAVENOUS INJECTION OF HISTAMINE

STUYVESANT BUTLER, M.D., WILLIAM A. THOMAS, M.D., AND HEYWORTH N. SANFORD, M.D. (BY INVITATION), CHICAGO, ILL.

It has been recognized for some time that during peptone and anaphylactic shock and thrombocytopenic purpura there occurs a simultaneous clumping out of platelets and vasodilatation. Theoretically this dilatation occurs because platelets are no longer available for the removal of free histamine from the blood, and this histamine is the cause of the vasodilatation. Some investigators, however, believe that the platelets themselves are a source of some of the body histamine.

We have attempted to discover whether a continuously high level of histamine in the blood for many hours will materially effect the qualitative platelet count either by driving platelets out of the free circulation or by causing their increase in an attempt to overcome the increased histamine concentration.

Fifteen patients who received intravenous injections of histamine for migraine were used. Blood was drawn before the onset of each injection and six hours later, toward the end, using the technique of Sanford and Leslie. Blood was collected in chilled syringes, counted on a chilled chamber, and centrifuged in waxed chilled tubes. The coagulation times for whole blood, cell-free plasma, and platelet-free plasma were determined. Hematocrit and platelet counts were made simultaneously.

The histamine was given at a constant rate of 20 drops per minute in 500 ml. of normal salt solution, but the amount of histamine was almost doubled with each injection, 0.2 mg. being used for the first injection and 2.0 mg. of histamine base for the last.



*Results.—*

1. There was a definite shortening of the coagulation time in most cases which in total varied from 1.9 minutes to 1.0 minutes.
2. There was a correlation between the qualitative platelet activity and the coagulation time.

#### 47. THE EFFECT OF CHRONIC ANEMIA ON RENAL FUNCTION AS MEASURED BY INULIN AND DIODRAST CLEARANCES

WILLIAM C. LEVIN, M.D. (BY INVITATION), RAYMOND GREGORY, M.D., AND  
ALENE BENNETT, B.A. (BY INVITATION), GALVESTON, TEXAS

This study has been made as a means of evaluating renal function quantitatively in patients with chronic anemias. Inulin and diodrast clearances were determined in fourteen patients; a total of twenty separate studies were made.

In five patients with pernicious anemia, nine renal clearance studies were obtained. The average glomerular filtration rate was 73 ml. per minute as compared with a control average of 119 ml. per minute. The average renal plasma flow in the patients with pernicious anemia was 419 ml. per minute (control average, 640 ml. per minute). The average effective renal blood flow in these patients was 617 ml. per minute (control average, 1050 ml. per minute). These results reflect very definite decreases in glomerular filtration rate, renal plasma flow, and effective renal blood flow. However, the most striking abnormality is evident in the effective renal blood flow.

Eight studies in six patients with sickle cell anemia revealed an even greater reduction of glomerular filtration rate, with an average of 60.2 ml. per minute. The average renal plasma flow in this group was reduced to about the same degree as in the patients with pernicious anemia with an average of 398 ml. per minute. The effective renal blood flow was determined in only four instances; hence, the average of 386 ml. per minute is not comparable.

It is significant that the effective renal blood flow was definitely increased in two patients with pernicious anemia after remissions had been induced following the institution of specific therapy.

Chronic anemia may be associated with decreases in glomerular filtration rate, renal plasma flow, and effective renal blood flow. The impairment in glomerular filtration rate is more marked in patients with sickle cell anemia than in patients with pernicious anemia. There is a tendency for recovery of these functions toward normal in patients with pernicious anemia as a remission develops.

#### 48. THE TREATMENT OF EXPERIMENTAL UREMIA BY INTESTINAL LAVAGE

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BALTIMORE, Md.

With the marked interest in peritoneal irrigation as a treatment of uremia, it was felt advisable to explore further the possibilities of intestinal irrigation despite a few unfavorable reports in the literature.

Isolated high intestinal loops (Thiry-Vella fistulas) were prepared in dogs and the intestinal continuity restored by end-to-end anastomosis. Eleven to twenty-eight days later a bilateral nephrectomy was performed. Five control dogs so treated died in from 52 to 104 hours following nephrectomy (average, seventy-three hours) and the terminal blood urea nitrogen levels varied from 145 to 265 mg. per cent (average, 193 mg. per cent). Six experimental dogs so treated, but, in addition, submitted to irrigations of the intestinal loop, died in from sixty-three to ninety-nine hours following nephrectomy (average, eighty-one hours) and the terminal blood urea nitrogen levels varied from 89 to 229 mg. per cent (average, 144 mg. per cent). It is thus seen that the duration of life in the irrigated dogs was not appreciably lengthened, probably because of a severe disturbance in the electrolyte pattern of the blood, which was only slightly relieved by calcium gluconate.

However, the average terminal blood urea nitrogen level was lowered by about 26 per cent. Furthermore, during the eighteen irrigation procedures to which the six experimental dogs were submitted, the blood urea nitrogen level was kept constant or was lowered in eight, or 45 per cent. The amount of urea nitrogen removed varied from 1.3 to 14 mg. per inch of intestine irrigated per hour. Best results in this regard were obtained with hypertonic fluids.

*Conclusion.*—Irrigation of intestinal loops will remove fairly large amounts of urea but does not appreciably prolong the life of the animals tested.

#### 49. EXPERIENCES WITH THE MURRAY ARTIFICIAL KIDNEY

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At the 1947 meeting of the Central Surgical Association, Gordon Murray of Toronto described an artificial kidney with which he had had some success in the removal of nitrogenous products from anuric dogs and human patients. The principle was that of dialysis through a cellophane membrane. Blood was caused to flow through small-bore cellophane tubing which was immersed in a solution containing the dialyzable substances which it was desired to retain. The blood was removed from one iliac vein by a catheter inserted through the saphenofemoral junction and was allowed to return into the other iliac vein. Propulsion of the blood was obtained by a sterile pump arrangement operated by an electric motor. The dialysate was maintained at body heat and was agitated by a suitable mechanical device. A trap was provided to prevent air embolism.

We prepared an apparatus similar to that of Murray. The dialysate solution was that of Kolff of Holland. It had the following composition: NaCl, 0.6 per cent;  $\text{NaHCO}_3$ , 0.2 per cent; KCl, 0.04 per cent; and glucose, 1.3 per cent. We have used the apparatus on ten nephrectomized dogs and two anuric patients. Approximately 2 Gm. of nonprotein nitrogen were removed each

hour from dogs whose blood nonprotein nitrogen was elevated to 100 to 200 per 100 milliliter. The chief adverse effect was related to the use of heparin, which is necessary to maintain the fluidity of the blood.

The first patient was a man, aged 21 years, with greatly diminished renal function due to repeated episodes of hemolysis. The artificial kidney was attached for eight hours, during which time his blood was dialyzed against 120 liters of solution. The initial nonprotein nitrogen was 260 mg. per cent. Although 27 Gm. of nonprotein nitrogen were recovered, the final nonprotein nitrogen of the blood showed a paradoxical increase to 294 mg. per cent. The run was discontinued because of serious bleeding around one of the catheters. The patient expired in pulmonary edema twelve hours after the apparatus had been disconnected.

The second patient was a man 52 years of age, with acute hepatitis and urinary suppression. He had been comatose for two days. It was questioned if the apparatus might serve as an "artificial liver" as well as an "artificial kidney." The apparatus was operated for twenty-nine and one-half hours; 81.6 Gm. of nitrogen were removed, the blood nonprotein nitrogen falling from 129 to 89 mg. per cent. There was no change in the blood sugar level, the chlorides rose from 365 to 427 mg. per cent, and the calcium reached the very low level of 3.5 mg. per cent. Fifty milliliters of 10 per cent calcium gluconate had been given to combat this calcium loss. At no time did the patient show clinical tetany. He expired eight hours after the run. No autopsy was obtained.

It appears that an apparatus of this type is effective to a limited extent in removing nitrogenous products. There is danger of hypocalcemia if the dialysate formula of Kolff is used.

## 50. LIPOID NEPHROSIS

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Clinical and biochemical studies of forty-one children with lipoid nephrosis are evaluated. Twenty-six of the children are alive; nineteen have been entirely well for one to sixteen years, including three who had minimal hematuria on various occasions. Three have been well for less than a year and four still have active disease. Of twenty-two children who had received sulfonamides for infections, only three have died. Necropsy was done on ten of the fifteen children who died. Minor glomerular changes were seen in the kidneys of four children not known to have had hematuria. Pronounced glomerular changes occurred in two children who had minimal hematuria. Of the four children who did not show any glomerular changes, two had minimal hematuria on several occasions. Pathologic changes in the liver were minimal unless acacia had been given.

Laboratory determinations revealed no significant alteration of the prothrombin time. Most of the increase in the blood fats was in the cholesterol fraction. The percentage of cholesterol esters was not decreased. In case of recovery, the cholesterol returned to normal values at a slower rate than the serum albumin. Blood fats reached values exceeding 5 Gm. per 100 ml. in some instances. No "critical edema level" for albumin was found. We have seen children free from edema with albumin values below 1 Gm. per 100 ml. and others with edema with values of 2.0 Gm. per 100 ml. The sedimentation test was a reliable guide in determining the course of the disease. Electrophoretic analyses of thirty plasma samples revealed uniformly low albumin values.

while the most pronounced change was the increase of the alpha 2 and beta fractions of globulin. During recovery, albumin increased, but the gamma globulin was slower in attaining normal values than the other fractions. No correlation was found between changes in the sedimentation rate and any individual protein fraction.

Choline citrate (0.8 Gm. each day) given to seven children for prolonged periods failed to produce essential changes in the blood fats or definite clinical improvement.

Periods of sudden change in edema were found to be critical times for the nephrotic child. Two children developed cardiac failure after the injection of large volumes of sucrose.

Early diagnosis is important, for treatment is most effective in the early stage.

The prognosis is good since the advent of chemo- and antibiotic therapy and is not impaired by prolonged duration. Growth of the children who recovered was good.

*(To be concluded in the December issue.)*

# COLLOIDAL GOLD CONTAINING THE RADIOACTIVE ISOTOPE $\text{Au}^{198}$ IN THE SELECTIVE INTERNAL RADIATION THERAPY OF DISEASES OF THE LYMPHOID SYSTEM

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THE use of colloidal sols containing radioactive manganese in selective internal radiation therapy of diseases of the lymphoid system has been reported in an earlier communication.<sup>1</sup> In the report it was stated that other isotopes might be of therapeutic interest. More recently the release of radioactive isotopes from the chain-reacting uranium pile prompted us to consider the use of a different isotope since  $\text{Mn}^{52}$  was not available in therapeutic amounts from this source and since the wide choice of isotopes permitted a more favorable selection to be made. In making an appropriate choice it was required that the half life, radiation, and specific activity be suitable (with absence of long-lived contaminants and toxic effects), that the production cost be low, and that the chemical and biological behavior be familiar. These requirements were best met by the radioactive isotope of gold  $\text{Au}^{198}$ . In this communication the suitability of this isotope for therapeutic use will be discussed, and methods of preparation of the sol, dosage determination, and administration will be described. Clinical results are reserved for a separate communication now in the course of preparation.

## HALF LIFE AND CONTAMINANTS

The half life of  $\text{Au}^{198}$  is 2.73 days.<sup>2</sup> We find that this rate of decay is ideal in therapy since the patient receives three-quarters of the radiation from a given dose in about five and one-half days. The therapist is then in a position to give additional treatment or not, at his own discretion. This permits rather good control of the radiation which is not possible in the case of longer-lived isotopes.

Practical considerations prevent the use at this time of isotopes of much shorter half life than  $\text{Au}^{198}$ . As decay rates materially increase, transportation must become increasingly rapid, and the possibility of preparing the colloid in one laboratory for delivery to another qualified center for administration is lost. Furthermore as the period of radiation decreases, biologically effective doses must increase, necessitating more elaborate precautions for the protection of the worker. Although not established there is reason to believe

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that the incidence of radiation sickness will increase as more intense doses of radiation are delivered over shorter periods of time.

Before any new radioactive isotope becomes acceptable for therapeutic use, the amount of long-lived contaminants present should be investigated. In the case of gold it is fortunate that no long-lived isotope of this element is known. Thus one must be concerned only with the presence of foreign elements in the original gold from which the isotope is prepared. Those most likely to be present are silver and copper. Traces of silver may give rise to the 225 day isotope of this element. For this reason a decay study was made on a sample of the gold containing the isotope. The decay was first investigated by following the gamma radiation with a Lauritsen electroscope; later the decay was followed by measuring the activity with a Geiger-Müller counter which is predominantly sensitive to the beta rays. It was found that the decay curve was strictly exponential for well over ten half lives at which time the activity became too weak to follow. This is a "clean" isotope only because gold of suitable purity was activated in the first place.

#### CHEMICAL AND BIOLOGICAL BEHAVIOR

The chemical behavior of gold is as familiar as that of most elements and the preparation of colloidal suspensoid sols of this element is extremely simple. Such sols are stable and will withstand repeated autoclaving at 120° C. They can be concentrated by boiling, and a reasonable amount of dissolved salts does not appear to affect the stability. This is in definite contrast to our experience with sols of manganese oxides which must be dispersed under sterile conditions since they do not withstand subsequent sterilization.

The biological behavior and toxicology of ionic gold are well known. The literature on colloids is less complete. However, the distribution in the rat of intravenously administered colloidal gold sulfide has been studied.<sup>3</sup> Our own investigation<sup>4</sup> has shown that, in the human being, particles of intravenously administered colloidal metallic gold are phagocytosed by reticulo-endothelial cells, with a higher concentration in the spleen than is obtained with manganese. If we assume that it is desirable to irradiate this organ rather completely in most instances the latter feature is a desirable one. In the present case the toxic effects of ionic gold present no problem. Due to the insolubility of metallic gold blood levels are low following its deposition in the body. An additional safety factor is achieved by limiting the amount of gold to 4 mg. or less per dose. In the course of considerable clinical experience we have never observed any effects which might be attributed to gold toxicity.

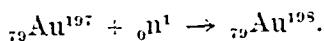
#### RADIATION EMITTED

$\text{Au}^{198}$  emits negative electrons with a maximum energy of 0.78 mev and two gamma rays with an energy of 0.28 and 0.44 mev have been found.<sup>2, 5</sup> Although a hard 2.5 mev gamma ray is reported it is not intense enough to make shielding difficult. Two inches of lead give ample protection for the largest amounts involved and considerably thinner shields suffice in many in-

stances. The gamma rays are penetrating enough to permit external tracing of activity in the body of the patient by the use of a directional Geiger-Müller counter. Recent studies would indicate that some features about the disintegration of  $\text{Au}^{199}$  still require further clarification.<sup>5</sup>

#### PRODUCTION OF RADIOACTIVE GOLD

The isotope is provided by the Atomic Energy Commission and is prepared by exposing metallic gold to the flux of slow neutrons in the chain-reacting pile at Oak Ridge. The reaction is



The simultaneous factors of abundant parent isotope, high capture cross section, and high neutron flux place this isotope in the high range of specific activities produced by the pile. It is this which permits such high and therefore therapeutically effective radiation doses to be administered in such small amounts of gold. At the present time the limitation in production is set primarily at quantities which may be safely shipped. Even with this limit the cost is roughly only two to five dollars per dose.

#### PREPARATION AND ADMINISTRATION

The chemical procedure in the case of radioactive gold is extremely simple. No radiochemical separation is required. It is merely necessary to convert the metallic gold to a solution of gold chloride (probably aurochloric acid) and to reduce this once more to metallic gold in alkaline solution by adding ascorbic acid. The presence of gelatin, although not essential, increases the reliability of the procedure.

Gold foil (16 mg.) containing radioactive gold is transferred to a long-necked Kjeldahl flask provided with a female ground joint. This is supported behind two-inch lead bricks and is viewed in a mirror. A few drops of aqua regia are sufficient to dissolve the metal. The solution is then evaporated to dryness. Since high temperature decomposes the gold salt, the flask is evacuated by inserting a male ground joint connected to a vacuum pump. The flask is heated with a water bath and because of its rounded shape safely withstands evacuation. After drying is complete, 50 c.c. of pyrogen-free water and  $\frac{1}{2}$  c.c. of Knox P-20 gelatin\* are added. The solution is made alkaline by adding 1 to 2 drops of concentrated KOH. A solution of 25 mg. ascorbic acid in 10 c.c. of water is then added; a deep red colloid forms instantly. In all operations the use of long pipettes and tongs provides added safety for the worker. Individual doses are dispensed from a simple pipette constructed from a 50 c.c. Pyrex graduated centrifuge tube of the conical type. The dispensing tip is attached to the bottom of the tube. Its long S shape permits withdrawal from the long-necked flask while the pipette rests behind the protecting lead shield. The open top is drawn down to a small diameter and is connected through a rubber tube to an external syringe by which the pressure may be controlled

\*Furnished by Dr. D. Tourtellotte of the Knox Gelatin Protein Products Co., Camden, N. J.

from outside the shield. The volume of liquid may be observed in the mirror. Doses are dispensed into penicillin bottles which are placed in lead-carrying shields. These provide protection during autoclaving and local transportation.

Intravenous administration is facilitated by the use of a saline infusion. The saline solution is run into the cubital vein in the usual manner and after satisfactory flow is established the sol is poured into the solution by the use of tongs. This technique permits the maintenance of a safe clearance at all times.

#### THE DOSAGE UNIT

The radioactivity of each dose is determined by measuring the gamma ray intensity at a distance of 1 M., using a Lauritsen electroscope.\* Measurements are made on an ordinary laboratory bench which introduces minor uncertainties in the radioactivity of samples of variable size and shape due to scattering and to absorption effects. However, studies have shown that the accuracy is sufficiently good for therapeutic work. Sufficient material must surround the sample to remove all beta rays. This is achieved by placing the sample container in a large glass beaker.

Pending the establishment of accurate millicurie standards of radioactive gold it was decided to adopt an empirical dosage unit. This was the amount of radioactive gold which produces a discharge rate of 0.5 small division per minute on our electroscope. The radium discharge rate determined by the manufacturer for this instrument was 2.14 small divisions per minute per milligram of radium at 1 meter. This figure should suffice to compare our instrument with others from this source. However, the lack of sufficient information concerning the technique of their measurement makes the value unreliable in absolute amount.

Before the absolute decay rate for a given sample of  $\text{Au}^{198}$  can be established with certainty it will be necessary to investigate the decay characteristics of the isotope further. However, if it can be assumed that the two gamma rays of  $\text{Au}^{198}$  are emitted in cascade at each disintegration and that the effect on the electroscope of each quantum is proportional to the energy of the quantum, it may be estimated from the response of the instrument to accurately calibrated radium needles of known filtration that one unit is roughly 0.3 millicuries. This estimate is subject to a number of uncertainties. In any event, conservative initial dosage is recommended in order that the response to radiation can be established to the satisfaction of the therapist.

#### SUMMARY

Colloidal gold containing the radioactive isotope  $\text{Au}^{198}$  has been used in therapy of diseases of the lymphoid system. The material is obtained at low cost from the chain-reacting pile at Oak Ridge. The isotope has an ideal half life and good radiation characteristics. The chemical procedures are simple, and extremely stable colloids can be prepared. Toxicity effects due to gold are

\*Manufactured by the Fred C. Henson Co., Pasadena, Calif.



not encountered. The technique for preparation, dosage measurement, and administration is described. Clinical results are reserved for a later communication.

We wish to thank the Isotopes Branch of the United States Atomic Energy Commission and the cooperative personnel of the Clinton Laboratories who made possible the use of the radioactive material investigated in this research.

#### REFERENCES

1. Hahn, P. F., and Sheppard, C. W.: Selective Radiation Obtained by the Intravenous Administration of Colloidal Radioactive Isotopes in Diseases of the Lymphoid System, *South. M. J.* 39: 558-562, 1946.
2. Diemer, G., and Groendijk, H.: Some Measurements on the 66 Hour Period of Gold, *Physica, 's Grav.*, 11: 396-400, 1946.
3. Block, Walter D., Buchanan, O. H., and Freyberg, R. H.: Metabolism, Toxicity, and Manner of Action of Gold Compounds Used in the Treatment of Arthritis. V. A Comparative Study of the Rate of Absorption, the Retention, and the Rate of Excretion of Gold Administered in Different Compounds, *J. Pharmacol. & Exper. Therap.* 82: 391-398, 1944.
4. Sheppard, C. W., Wells, E. Buist, Hahn, P. F., and Goodell, James P. B.: Studies of the Distribution of Intravenously Administered Colloidal Sols of Manganese Dioxide and Gold in Human Beings and Dogs Using Radioactive Isotopes, *J. Lab. & Clin. Med.* 32: 274-286, 1947.
5. Jnananda, S.: Radioactive Isotope of Gold,  $^{198}\text{Au}$  and Low Energy Range of Its Spectrum, *Physical Review* 70: 812-814, 1946.

## DIRECT INFILTRATION OF RADIOACTIVE ISOTOPES AS A MEANS OF DELIVERING IONIZING RADIATION TO DISCRETE TISSUES

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ELSEWHERE<sup>1, 2, 4</sup> we have described the use of radioactive colloids of  $\text{Mn}^{54}\text{O}_2$  and  $\text{Au}^{198}$  in an attempt to radiate specifically the lymphoid-macrophage system. The  $\text{MnO}_2$  sols were found to be gradually liberated, with evidence of secretion by way of the biliary tract. In the case of the gold sols, however, it was found by use of a directional type of counter<sup>3</sup> that the radioactive material remained in the tissues in which it had been deposited (that is, chiefly the liver and the spleen) for as long as measurements could be made.<sup>5</sup> Because of the chemical characteristics of metallic gold it was felt advisable to determine whether the insolubility of gold colloids in body fluids could be used to advantage in direct infiltration of neoplastic tissue.

When radium needles or radon seeds are implanted in tissue in order to provide localized radiation, it is necessary to filter the alpha and beta emanations in order to avoid too intense radiation immediately about the needle or seed itself. Dependence is then placed on the cross fire of gamma radiation to the affected tissue, but some radiation of the adjacent normal structures cannot be avoided. In utilizing the radiation from colloidal sols directly infiltrated into the tumor tissue, many millions of "point sources" of radiation are involved in emitting beta rays diffusely throughout the injected volume of tissue. Since the mean free path of the beta ray is of the general order of a millimeter, any considerable irradiation of normal surrounding tissue is avoided. In the case of either  $\text{Au}^{198}$  or  $\text{Au}^{199}$  isotopes one is not too greatly concerned with the gamma radiation per se since the ionization produced is not of great consequence in comparison with that derived from the beta radiation. However, the gamma radiation is of particular value in enabling the investigator to measure the localization and relative degree of concentration of infiltrated material.

Certain metastatic lesions are so vascular that injection results in effecting an intravenous administration of the colloidal particles, as will be seen in at least one instance below.

Also, some tumor tissue has been found to be so friable or otherwise constituted that it will not contain even relatively small volumes of fluid. In such instances the procedure as outlined in the foregoing becomes untenable. Under such conditions it is felt that resort to other procedures must be taken in order to establish the radioactive material at the desired site. Preliminary investigations are under way to determine whether the propensity for silver to become

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fixed to tissues might be employed to circumvent this problem.<sup>7, 9</sup> Several possible reactions are involved: (1) Reduction of the radioactive silver when injected as the  $\text{Ag}^*\text{NO}_3$  salt to metallic silver with relatively permanent fixation by the tissue by the presence of reducing materials such as glutathione and cevitamic acid; (2) precipitation of the silver as a chloride by interaction with tissue chlorides; and (3) combination of the silver with the tissue proteins to form relatively insoluble compounds. Silver colloids, especially that of the oxide, also seem promising at the present writing.<sup>9</sup>

Possibilities involved in the use of radioactive silver are legion. Topical application of radioactive silver nitrate is a logical and reasonable approach to irradiation of superficial lesions.

Because  $\text{Ag}^{111}$  is a pure beta emitter, it is not possible to apply adequately external measurement apparatus for determination of the localization or intensity of radiation from the material deposited deeply in the tissues. This may be circumvented in part by adding small tracer quantities of long-lived  $\text{Ag}^{108, 110}$ , but such a procedure must be considered carefully because little is known regarding the rate of removal of silver from tissues under such conditions. In the case of superficial tumors which, following radiation, can be reliably expected to slough off, such a procedure might be justified. The experimental preliminary studies demonstrating the localization of silver in tissues will be reported elsewhere.<sup>7, 9</sup>

#### METHODS

The preparation of the colloidal sols has been described earlier.<sup>1, 4</sup> Ordinarily the gold sols for intravenous administration were made up to contain 2 Vanderbilt units (V. U.) per milliliter. Such concentrations of radioactivity were usually found to be too low for infiltration work because of the necessity of injecting relatively large volumes of solutions. Therefore the gold colloids were concentrated by boiling so that 5 or more units per milliliter were obtained. The stability of these colloids allows this to be done successfully. It might also be equally practicable to make up the colloid in a smaller volume of solution originally, although difficulties due to "clumping out" of the gold have been encountered occasionally upon attempting to do this.

The "Vanderbilt unit" represents what we estimated at an earlier date to be an approximation of a millicurie and it is an arbitrary unit of dosage. It is now felt to represent nearer  $\frac{2}{3}$  millicurie. The material is measured at one meter with adequate shielding of beta radiation so that the gamma radiation from  $\text{Au}^{198}$  only is read on a Lauritsen, radium calibrated electroscope.\* Under such conditions a single "Radiation Unit" obtained from Oak Ridge, consisting of 16.0 mg. of gold foil, which has been exposed to a flux of slow neutrons, would have on an average of about 120 V. U. at time of removal from the uranium pile. It has been found that approximately 40 V. U. of  $\text{Au}^{198}$  administered by vein to an adult of average size may result in a remission in chronic lymphocytic leukemia.<sup>8</sup> However, it must be kept in mind that there is a considerable amount of individual variation in response to such therapy.

In the cases of discrete encapsulated lesions, the colloid was administered in the same manner as would be employed for infiltration of a novocain solution. The long needle, preferably of small caliber was inserted the maximum desired distance into the tissue and the sol released gradually as the needle was withdrawn.

Following infiltration, frequent measurements were made using the directional counter.<sup>3</sup> This instrument consists of two gamma sensitive Geiger-Müller tubes enclosed in about an inch-thick cylindrical lead shield. One of the counters is positioned opposite a removable

\*Obtained from the Fred C. Henson Co., Pasadena, Calif.

port whose opening may be varied from one-fourth to three-fourths inch. The other is completely shielded by the lead. The output of these tubes is fed into two counting rate meters which, by an appropriate circuit, are made to subtract. The degree of directionality obtained is quite high. One is able to find the edge of the liver or spleen with ease, and maintenance of injected material in nodes or tumor masses can be readily followed. The whole shielded tube is mounted in a carriage which permits ready translation on all axes as well as rotation.

#### CLINICAL OBSERVATIONS

E. R., a 28-year-old white man, had been treated for Hodgkin's disease at various times since November, 1945, with x-ray, radioactive colloidal manganese dioxide, nitrogen mustard, and radioactive colloidal gold, respectively. The patient showed good general response to radiation therapy except that there were two persistent enlarged lymph nodes in the right posterior cervical chain. The larger of these measured 3 by 2 by 1.5 cm. and the smaller measured 1 by 1 by 1 cm.; 0.25 ml. (1 V.U.) of colloidal radioactive gold sol was injected into the former and half that quantity into the latter by infiltration. The patient complained of a burning sensation at the site of injection for about one minute, after which the pain ceased. Daily examinations during the ensuing week revealed no swelling, redness, or increased heat in these areas, although there was some tenderness on pressure. One week after the injection there was no decrease in the size of either node. At this time the larger node was infiltrated with 1.5 ml. of the gold sol containing 5 V.U. and the smaller with 0.3 ml. containing 1 V.U. About three hours after injection the patient complained of aching pain in these areas. Redness, swelling and tenderness developed, reaching a maximum in about twelve hours, and gradually subsided. The patient was discharged and was not seen for four months at which time he was readmitted to the hospital. There was an area measuring about 2 by 2 cm. in the right lateral cervical area directly over the site of gold injection which showed a bluish discoloration. There was a slight tenderness in this area and a small pea-sized, nontender, firm node was palpable just inferior to the area of discoloration. No other lymph node enlargement was observed.

H. H., a 57-year-old white man, had had a long standing bronchiectasis and known generalized lymph node enlargement of three years' duration. On Mar. 6, 1946, biopsy of an axillary node revealed lymphosarcoma. Intravenous therapy with radioactive colloidal manganese dioxide was begun and an enlarged lump over the left clavicle diminished somewhat in size as did most of the lymph nodes. There was marked subjective improvement. He was given occasional intravenous injections of colloidal  $\text{MnO}_2$  and later of colloidal gold. On Feb. 12, 1947, he was admitted to the hospital for blood transfusion and at this time the supraclavicular mass measured 14 by 12 cm. and was elevated 3.5 cm. above the surface of the surrounding skin (Fig. 1). Seven V.U. of colloidal radioactive gold in 7.0 ml. of solution were infiltrated diffusely into the center of the mass. The patient noted a sensation of constriction in the area for several hours, but no other symptoms were subjectively felt and over a period of twenty-four hours there was no tenderness, increased heat, or redness. A week later there was a definite decrease in the size of the mass centering about the injected area and it was softer. Measurement showed the mass to be 12 by 10

centimeters. Use of the directional counter for measurement of gamma ray activity showed good localization of the radioactivity in the mass. At this time an additional 12 V.U. of sol in 9.0 ml. of solution were infiltrated into the tumor tissue in the upper mid-portion; the patient again complained of a feeling of



Fig.1.—Fibrosarcoma prior to treatment.



Fig. 2.—Fibrosarcoma four weeks later.

tightness and constriction but nothing else. Another 14 V.U. were infiltrated on Feb. 26, 1947, and an additional 7 V.U. on Mar. 19, 1947.

The infiltrated mass had completely disappeared at examination on April 1, 1947 (Fig. 2).

G. S., a 35-year-old white woman (gravida ii, para i, abortus i), was admitted to the gynecologic service May 5, 1947. Five months previously she had received a supravaginal hysterectomy and bilateral salpingo-oöphorectomy for vaginal bleeding. She was found at this admission to have a Group 4 squamous cell carcinoma of the cervical stump (pathologic grade 3). Pelvic examination showed an asymmetrical, nodular cervix which showed gross evidence of carcinomatous slough and growth. The lesion measured 3.5 by 3.5 cm., with marked necrosis of the right posterior lip and central portion. The tissue was very friable and bled easily on manipulation. Infiltration of the parametria was found all the way to the pelvic wall on both sides. On May 8, 1947, 11 V.U. of radioactive colloidal gold sol (3.5 ml.) were injected into the left lateral portion of the cancer mass. The right half was not infiltrated to serve as a control. A lead-shielded 2 ml. syringe with a 22-gauge needle was used for the injection in a manner such as employed for local anesthesia. A vaginal pack was inserted and all towels and operative packs were collected for determination of radioactivity lost by leakage. On May 9 the pack was removed and a reading on the Lauritsen electroscope showed that there was no significant (less than 0.5 V.U.) loss of material.

Observation of the site of injection showed no appreciable change until May 15 when two observers noted a slight area of redness on the left as contrasted with the right. Biopsy was taken of the injected and of the control areas. No difference was noted except some increase in mitoses in the untreated area which was not considered significant. On May 10, 13, and 18 measurements by means of the directional differential counting rate meter<sup>1</sup> showed that the radioactive material was well localized in the pelvis and neither the liver nor spleen showed any radioactivity.

On May 21, 44 V.U. of gold sol (13 ml.) were infiltrated into the entire carcinomatous mass. There was a moderate degree of bleeding during the procedure and some of the radioactive material was seen to ooze from the injected site. It was felt that 13 ml. were too large a volume of solution for this size of growth. Vaginal packs removed the following day, as well as packs saved at the time of injection, showed a loss of 24 V.U. of material. On May 23, localization studies with the directional counter showed very small amounts of activity in the hepatic and splenic areas, but most of the radioactivity was demonstrable in the pelvis. Similar findings were noted on May 25. The patient showed no systemic reaction to the radioactive material and was discharged on May 29, 1947, to be followed in the outpatient clinic. The patient died at home on July 13, 1947.

M. B., a 20-year-old unmarried colored woman (gravida iii, abortus iii), while under treatment for secondary syphilis was found to have squamous cell carcinoma of the vagina. On May 22, 1947, the patient was found to have a friable, fungating mass approximately 4 by 6 cm. in the posterior vaginal wall. On May 28, 60 V.U. of radio gold sol were infiltrated. Operative packs, as well as the vaginal pack removed the following day, showed a loss of 57 V.U. of gold,

leaving a negligible amount in the injected mass. However, on May 31 what material was left in the tumor was found to be well localized by means of the directional counter.

On June 5, 1947, 65 V.U. of radio gold sol (4 ml. of solution) were infiltrated into the carcinomatous mass, but seepage collected in the packs showed a loss of 50 V.U., leaving only 15 units in the tissue. There was no systemic reaction to the radioactive material.

It was apparent that the extreme friability of this tissue precluded the retention of the gold sol. The patient was later treated with interstitial implantation of radium.

E. T., a 62-year-old colored woman (gravida i. para i), was found to have a far advanced (group 4) squamous cell carcinoma of the cervix. The patient previously had received deep x-ray and radium therapy with no improvement. There had been several episodes of vaginal bleeding and the patient was admitted on May 11, 1947. Pelvic examination showed marked stenosis of the vaginal canal, obliteration of all vaginal fornices, and replacement of the cervix by nodular, necrotic cancer mass. On May 15, 16 V.U. of gold sol (3 ml. of solution) were infiltrated, and later measurement of the packs showed less than 1 V.U. lost by seepage. Observations with the directional counter on May 17 and 23 showed the radioactive material to be well localized in the pelvis, none being detectable in the hepatic and splenic areas. There was no systemic manifestation attributable to the radioactive material. Daily temperature spikes existing prior to the infiltration showed no change. The patient was discharged on May 28, 1947, to be followed in outpatient clinic. On the tenth day following discharge the patient reported a small hemorrhage of an hour's duration. No significant bleeding occurred in the six weeks following. It was the consensus that there was no significant change in the condition of the carcinomatous mass due to the radioactive material, possibly because of the low dosage employed. The tissue showed the ability to retain the material and it was felt that such local injection of radioactive inert material might offer an approach to the problem of hemostasis in bleeding of far advanced cases of cancer of the cervix.

C. S., a 29-year-old white man, had been under treatment for chronic lymphocytic leucemia for the past year. The patient originally had shown marked hepatosplenomegaly and extensive lymph node enlargement. There were numerous subcutaneous lesions seen on the forehead. These were violaceous in color and were nontender, varying in size from  $\frac{1}{2}$  to 2 cm. in diameter. In addition there were a number of pinkish-tan areas of infiltration in the skin of the trunk and of both arms, varying in size from 0.5 to 2.5 cm. and averaging about 1 cm. in diameter. While under intravenous treatment with the radioactive manganese dioxide colloid, there was a diminution in the size of the liver and spleen and a reduction in the white cell count to desired limits as well as good general subjective improvement. Nevertheless the subcutaneous lesions were unaffected over the period of a year and, if anything, were increasing in size and number. On Jan. 2, 1947, 0.15 ml. of colloidal radio gold (0.5 V.U.) was injected into each of the two nodules on the forehead. These nodules were carefully measured, together with two adjacent nodes left uninjected as controls.

Six days later the patient reported no subjective change since the injection except persistent itching in the injected nodes, but no redness, pain, or increased heat. Examination showed no change in the size of the nodes in the horizontal plane. Palpation, however, revealed a definite hollowing out effect in the center of each injected nodule. The nodes which had measured about 2.0 cm. in diameter were depressed and softened in an area measuring about 1.0 cm. in diameter. There was no redness or tenderness. At this time, 0.25 ml. (1.0 V.U.) was injected into each of three new nodules of about 2 cm. diameter on the forehead. The patient complained of a stinging sensation at site of each injection. The next week the three nodes injected were flat, although the outline of each was clearly distinguishable on the surface of the skin and the dimensions in the horizontal plane remained the same. There was a grayish-black discoloration of the skin which measured about 1.0 cm. in diameter immediately overlying the site of each injection. There was no redness, tenderness, swelling, or increased heat in any of these areas. At this time, 2.0 V.U. in 0.3 ml. of solution of colloidal gold were injected into each of the two most posterior nodules on the top of the head. The next week, on examination, the last two nodules injected were entirely flat. There was no pain or tenderness or local tissue reaction at the injected site.

The following week the patient was readmitted to the hospital for blood transfusion and supportive therapy. On this occasion some preliminary attempts at topical application were carried out. The gauze portion of several Band-Aids was soaked with radio gold in the form of the chloride and placed in position over several of the lesions on the upper back. Each Band-Aid contained about 2.0 V.U. of radioactive material. During the ensuing three days a small area of erythema began to develop in each of the treated areas and the patient was aware of a progressive increase in tenderness. On the third day the Band-Aids were removed. At that time each area of erythema measured approximately 3.0 cm. in width. The inflammatory reaction became progressively more marked and at the end of two weeks there was a crust of dry tissue measuring about 2.0 by 3.0 cm., this being surrounded by a ring of erythema of about 2 cm. width. The lesions at this stage were nontender, dry, and clean. During the following week the crusted portions of the lesions underwent desquamation, leaving underneath an area of smooth, pink, normal-appearing skin in which all gross evidence of leucemoid infiltration had disappeared.

On a number of later occasions subcutaneous nodules were infiltrated with colloidal radio gold, usually 1 V.U. of material being injected in each site. The reaction to the radiation was similar to that seen in the earlier trials.

When the patient reported on May 13, 1947, the most striking change was marked increase in the size of the lymphoid masses earlier found in the groin, that on the right measuring 16 by 8 cm. and that on the left 12 by 9 centimeters. Each mass was raised about 2 cm. above the surrounding skin. Both were firm and nontender. Fifteen V.U. of radio colloidal gold in 15 ml. of solution were infiltrated into the mass in the right groin. The transitory burning sensation disappeared after thirty seconds. Eight days later the skin overlying the injected mass showed a dark red discoloration but there was no increased heat or



tenderness in the region. The mass was slightly decreased in size, measuring 14 by 7 cm., and was definitely softer than the uninjected mass on the left. On this date the patient announced that he was leaving town for an indefinite period. When he was readmitted to Vanderbilt Hospital on July 1, 1947, it was found that the "right inguinal mass had diminished greatly in size while that on the left was slightly larger than previously," although the examining house officer did not make any actual measurements. The patient died as a result of hemorrhage into the peritoneum on August 3, 1947.

R. M., a 64-year-old white man with hypernephroma and metastases, first was admitted to Vanderbilt Hospital with the chief complaint of weight loss. The patient had been well until four months previously when he developed postprandial pain relieved by vomiting. There were also anorexia, epigastric fullness, flatulence, abdominal distention, and constipation. There was a weight loss of forty-five pounds in three months. The pain gradually became more severe and the patient weaker. There was an enlarged supraclavicular node as well as enlarged inguinal nodes. Retrograde pyelograms showed filling defects in the calices of the right kidney. A biopsy of the supraclavicular node revealed a hypernephroma metastatic to the node. On Jan. 15, 1947, 45 V.U. of radio colloidal gold diluted in 400 ml. of saline were administered intraperitoneally by slow drip. The patient tolerated the procedure well and complained of no burning or pain at the site of the injection or in the abdomen. During the ensuing five days repeated determinations of the localization of the radioactive material were made with the directional counter. These showed that the material was diffusely spread throughout the cavity and that the majority remained in the peritoneum until radioactive decay was nearly completed. Very little radioactivity could be demonstrated in the liver and none in the spleen, in which sites the gold would be expected to lodge if it were passed via the lymphatics of the diaphragm into the circulation.<sup>6</sup> At no time did the patient complain of any abdominal pain, nor did repeated examinations reveal any tenderness or spasm. The patient was followed in the outpatient clinic; his course was gradually downhill.

C. B., a 52-year-old white woman, had a seven months' history of hypernephroma. The patient had been treated with radioactive colloidal gold by vein on two previous occasions and that material was well localized in the liver, the spleen, and the remainder of the lymphoid-macrophage system, as shown by use of the directional counter.<sup>3</sup> On Feb. 5, 1947, the patient was given 20 V.U. of radioactive gold intraperitoneally in the form of the colloid. Three hours after the injection the patient complained of a severe diffuse abdominal pain. Examination revealed marked tenderness throughout the abdomen. The pain and tenderness gradually disappeared in the next twenty-four hours and the patient was discharged a day later. Repeated surveys with the directional Geiger-Müller counter during the next ten days showed a high degree of activity spread over the entire abdomen.

On a later occasion a metastatic lesion in the right occipitoparietal part of the scalp was directly injected by infiltration with 4 V.U. of colloidal gold. The

lesion was raised, round, and fluctuant and measured 4 cm. in diameter. The patient complained of no pain or burning following the injection, but subsequent radioactivity measurements over the area showed no localization at the site and suggested that the material was promptly removed by the blood stream.

C. S., a 66-year-old white man, was admitted to the hospital on May 14, 1947, with the chief complaint of "swelling of the left eye, trouble seeing and pain." About three months previously he had noted the first swelling on the left side of the nose and of the inner aspect of the left eye. There was also some visual difficulty and diplopia, and frontal headache without dizziness or fainting spells. There was no discharge or bleeding from the left nostril but during the previous month there had been obstruction of this nostril. There was a



Fig. 3.

Fig 3—Squamous cell carcinoma prior to local infiltration.



Fig. 1.

Fig 1.—Squamous cell carcinoma five weeks later.

weight loss of forty pounds in the previous six months. Three weeks prior to admission the patient reported to a physician who removed "a large amount of tissue" from the nostril and referred him to Vanderbilt Hospital.

A year prior to admission the patient had noted a lump in the throat which "cut off my wind" and produced dysphagia and soreness of throat with difficulty in speech. Shortly before this the patient had noted slight hemoptysis characterized by bright red blood on two occasions. On consulting a physician the patient was told he had a tumor of the throat and sent to Nashville where the tumor was biopsied and forty-three x-ray treatments given on successive days. At the end of this period symptoms entirely disappeared. Three months

before admission the patient noted gradual enlargement of lymph nodes on the left side of the neck as well as those in the inguinal region.

Of chief interest on examination was a pterygium on the left medial side of the eye with slight exophthalmos of the left eye. Along the left side of the nose and along the medial aspect of the left eye was a reddish-blue swelling which extended upon the frontal bone about 1.0 cm. above the eyebrow. The left eyeball was displaced laterally and there was moderate tenderness over the swollen area. The left nostril was filled with gray-colored tumor tissue. The tongue and hard palate were hyperemic and smooth, with mild tenderness. Biopsy showed the lesion to be a squamous cell carcinoma. (See Fig. 3.)

On May 14, the skin over the nose was prepared with iodine and alcohol and novocaine infiltrated into the tumor mass. Nine Vanderbilt units of colloidal radio gold were diffusely infiltrated into that part of the tumor overlying the nose. The tumor mass in the left nostril was also infiltrated with novocaine and 4.0 V.U. of gold were injected. The patient complained of a slight burning sensation in the injected area which disappeared in thirty seconds. On May 21, when the patient reported to the clinic, the external mass over the nose and eye was markedly reduced in size. However, two areas at the medial corner of the eye which were not injected were of the same size. The intranasal mass on the left had almost disappeared. A repeat biopsy was taken from the posterior nares. Radio colloidal gold was injected into the area around the medial corner of the left eye, a total of 11 V.U. in about 3.5 ml. of solution being used. An additional 3.5 V.U. were injected into the intranasal tumor on the left. (See Fig. 4.)

#### DISCUSSION

Direct infiltration of insoluble radioactive colloidal sols of gold is apparently a suitable manner in which to deliver a given amount of radiation to a discrete mass of tissue under certain circumstances.

X-ray can be delivered only from an external source and, except in superficial lesions, must traverse normal tissues. In tumors originating in deep tissues of the body, a considerable thickness of normal tissues must receive extensive irradiation in order to obtain effective dosage in the tumor. In fact, if the lesion is very resistant to irradiation and deeply seated in the body, effective treatment cannot be delivered by x-ray because of extensive damage produced in the intervening normal structures. In the case of injection treatment with radioisotopes, the treatment is from within the tumor toward the normal tissue. Because of the relatively short path of the beta radiations, considerable effect can be produced in the tumor tissue with relatively little damage to the surrounding normal structures. Injection treatment of deep tissue malignancies may eventually prove to be a very valuable agent in the treatment of cancer. Further experimentation with various malignant tumors and possibly other isotopes is necessary to put this form of treatment on a sound clinical basis.

It was somewhat surprising to us that in the two instances in which gold sols were given intraperitoneally the material remained in the peritoneum, since it had been shown that red cells tagged with isotopes when given by this route find their way rapidly into the systemic circulation by way of the

lymphatic system.<sup>6</sup> It would seem that where generalized diffuse radiation of the peritoneal cavity is desired, as perhaps in abdominal Hodgkin's disease, this might afford a reasonable means of accomplishing it.

In one of the patients with a scalp lesion metastatic to a hypernephroma, injection of the colloidal gold failed to show retention at the site of administration. At autopsy some weeks later it was found that these lesions were traversed by large sinusoids containing blood, and we feel that, in effect, in this case the result was similar to that which would have occurred with intravenous injection.

Most of the subjects treated were far advanced cases in which trial of materials whose correct dosage was unknown was felt justified. In every instance an attempt was made to err on the conservative side and, therefore, we feel that usually the patient was grossly undertreated. However, until more is known concerning the equivalent dosages for various isotopes and until sufficient empirical experience has been obtained to set the correct limits of dosage such a procedure probably will be necessary.

In patient M. B., with carcinoma of the vagina, the character of the tumor mass was such as not to hold injected fluid in a satisfactory manner. Packs removed after instillation and after twenty-four hours showed that practically all of the radioactive colloid had leaked out of this cauliflower-like tissue. Under such conditions it is apparent that the infiltration of gold is highly unsuccessful. We feel that another approach to such problems would be the instillation of  $\text{Ag}^{111}\text{NO}_3$ , in which the caustic action of the agent is such as to offer hope of tying it to the desired tissue. Under these conditions we might expect that the silver nitrate might undergo reduction by such compounds as ascorbic acid, glutathione, or any other naturally occurring reducing agent; it might react with the chlorides of the tissue fluids precipitating the  $\text{AgCl}$ ; or it might become bound directly to the tissue proteins. It is well known that in argyria the silver remains in certain sites for extremely long periods of time.  $\text{Ag}^{111}$  has a pure beta ray spectrum and a half life of seven and one-half days. It is made by transmutation from palladium and therefore has a maximum specific activity eliminating concern of toxic reactions.

The feasibility of use of  $\text{AgNO}_3$  by direct infiltration has been briefly tested in experimental animals to determine whether it remained at the site of injection.<sup>7, 8</sup> These experiments were carried out using  $\text{Ag}^{108, 110}$  made by neutron capture in  $\text{AgNO}_3$ . The half life is 225 days and therefore not suitable for human studies. The amounts used in such tracer studies were extremely small (about 57  $\mu\text{g}$   $\text{AgNO}_3$ ) so the conditions were comparable to what might obtain when using the carrier-free  $\text{Ag}^{111}$  in human therapy.

#### SUMMARY

1. Under certain conditions it is possible to obtain a high degree of selective radiation of discrete masses of tumor tissue by the direct infiltration of colloidal sols of radioactive gold. In general, the ultramicroscopic particles of gold remain in situ presumably because of their insolubility in body tissue fluids. As a result, a diffuse radiation is possible at any level of tissue ionization desired.

2. Unlike radium implants, it is not necessary to remove the vehicle supplying the source of radiation. Furthermore this material can be infiltrated into tissues which are inaccessible to x-ray and radium therapy, especially should this be feasible in the cases of inoperable tumors found during laparotomy.

3. Cellular structure or vascularity of a tissue may be such as to preclude the retention of solutions injected and the cauterizing properties of  $\text{AgNO}_3$  are suggested as an alternative, using the isotope Ag.<sup>111</sup>

We wish to express our appreciation for the cooperation of many members of the resident house staffs in Medicine, Obstetrics, and Surgery, as well as to the members of the Department of Pathology for their valued assistance. We are especially indebted to Dr. John C. Burch, Dr. Herbert Duncan, Dr. G. S. McClellan, Dr. W. David Strayhorn, Jr., and Dr. Albert Weinstein for their cooperation.

#### REFERENCES

1. Hahn, P. F., and Sheppard, C. W.: Selective Radiation Obtained by the Intravenous Administration of Colloidal Radioactive Isotopes in Diseases of the Lymphoid System, *South. M. J.* 39: 558, 1946.
2. Sheppard, C. W., and Hahn, P. F.: Retention and Excretion of Manganese Dioxide Dispersions Administered Intravenously to Humans, *South. M. J.* 39: 562, 1946.
3. Sheppard, C. W., and Weiland, Raymond: A Directional Geiger-Müller Counter Employing Differential Counting Rate Meters, *Rev. Scient. Instruments*. In press.
4. Sheppard, C. W., Wells, E. Buist, Hahn, P. F., and Goodell, James P. B.: Studies of the Distribution of Intravenously Administered Colloidal Sols of Manganese Dioxide and Gold in Human Beings and Dogs Using the Radioactive Isotopes, *J. LAB. & CLIN. MED.* 32: 274, 1947.
5. Jones, H. B., Wrobel, C. J., and Lyons, W. R.: A Method of Distributing Beta Radiation to the Reticulo-endothelial System and Adjacent Tissues, *J. Clin. Investigation* 28: 783, 1944.
6. Hahn, P. F., Miller, L. L., Robscheit-Robbins, F. S., and Whipple, G. H.: Peritoneal Absorption. Red Cells Labelled by Radio-Iron Hemoglobin Move Promptly From the Peritoneal Cavity Into the Circulation, *J. Exper. Med.* 80: 77, 1944.
7. West, Harold D., Elliott, R. R., and Hahn, P. F.: The Distribution and Localization of Radioactive Silver ( $\text{Ag}^{108,110}$ ) Following Injection and Topical Application in Experimental Animals. In preparation.
8. Hahn, P. F., Sheppard, C. W., Goodell, J. P. B., Francis, H. C., Motley, Lyle, Whiteleather, J. C., Jones, Edgar, Weinstein, Albert, and Strayhorn, W. David, Jr.: The Treatment of Various Blood Dyscrasias, Hodgkin's, and Other Malignant Neoplasms by the Intravenous Administration of Radioactive Colloidal Sols of Manganese Dioxide and Gold. In preparation.
9. Rouser, George, and Hahn, P. F.: The Preparation of Radioactive Silver Colloids. The Distribution of Salts and Colloids of Silver in Experimental Animals and in Human Beings Following Parenteral Administration. In preparation.
10. Sheppard, C. W., and Hahn, P. F.: The Use of Colloidal Radioactive Gold in Medical Therapy, *Federation Proc.* 6: 399, 1947.

# METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE

## XIV. THE UTILIZATION OF PANTOTHENIC ACID DURING LACTATION

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MANY studies of the metabolism of pantothenic acid have been made with normal and lactating animals, but little information is available concerning its role in human nutrition. Consequently, the Food and Nutrition Board of the National Research Council was not able to include this vitamin in its list of Recommended Dietary Allowances. Since animal experiments have indicated the importance of this vitamin in reproduction<sup>1,2</sup> and in the growth of young animals,<sup>4,5</sup> it is likely that it is important similarly in human nutrition. During studies of the utilization of nutriment from food by normal lactating women, pantothenic acid was determined in their food, milk, and urine during the first ten days post partum and during five-day periods at intervals during mature milk production. Other papers have presented the details of the selection of subjects and the organization of the study,<sup>6</sup> the dietary,<sup>7</sup> the method of expressing the milk,<sup>8</sup> and its contents of niacin, pantothenic acid, and biotin,<sup>9</sup> thiamine,<sup>10</sup> riboflavin,<sup>11</sup> ascorbic acid,<sup>12</sup> vitamin A and carotenoids.<sup>13</sup> The proximate composition,<sup>14</sup> lipid<sup>15</sup> and vitamin<sup>16</sup> contents of human placentas have been published as well as the utilization of ascorbic acid,<sup>17</sup> vitamin A and carotenoids,<sup>18</sup> thiamine,<sup>19</sup> riboflavin,<sup>20</sup> and niacin<sup>21</sup> by the nursing mothers. The utilization of biotin is discussed in a following paper.<sup>22</sup> As an adjunct to the study, the amounts of vitamins in the milk and urine of the mothers were determined before and after ten days in which multiple vitamin supplements were added to the diets. The effect of the supplement upon the utilization of vitamin C has been published<sup>23</sup> and its influence upon utilization of other vitamins will be discussed in papers now in preparation.

### PROCEDURE

The subjects of the study were multiparas selected during the first trimester of pregnancy on the basis of physical and laboratory examinations and records of having successfully nursed their other children. During all periods of study the women received diets comparable qualitatively but varying in the quantity of all foods, except milk, according to appetite.<sup>7</sup> Different menus were served each day of the five-day study periods and repeated during each period. Aliquot

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portions of each food as eaten were weighed into a composite which was accumulated for each five-day period of study. Both breasts were completely emptied by manual expression at the usual nursing intervals and samples taken for analysis from the complete twenty-four hour collection of milk. The entire excretion of urine during each twenty-four hours also was obtained for analysis.

Seven women were studied throughout the first ten days post partum. Values were obtained for three other women on their seventh days following delivery. Five of the seven women and four others were studied during seventeen five-day periods while they were producing mature milk.

#### METHOD OF ANALYSIS

The preparation of the milk samples and the general microbiologic procedure of analysis have been described.<sup>9</sup>

Aliquots (400 Gm.) of ground, mixed food composites were homogenized in a Waring Blendor, transferred quantitatively to a 500 ml. volumetric flask, and diluted to volume. Duplicate 5 ml. aliquots (representing 0.6 to 0.7 Gm. of dry food material) were pipetted into one-liter Erlenmeyer flasks and 400 ml. of distilled water added to each. The flasks were autoclaved for fifteen minutes at 15 pounds pressure. When cool, 1 Gm. of mylase P\* in 50 ml. of acetate buffer at pH 4.6 was added to each flask, after which they were incubated at 37° for twenty hours. After heating to 100°, the flasks were cooled, their contents adjusted to a pH of 5.3 to 5.7, diluted to 500 ml., and filtered through Whatman No. 42 paper. The filtrate was diluted to a concentration between 1:100 and 1:200 for assay. The values obtained were corrected for the pantothenate in the enzyme.

Urine was filtered, if necessary, brought to a pH of 6.5 to 7.0, and diluted to a concentration 1:100 and 1:200 for assay.

#### RESULTS

The twenty-four hour milk and urine volumes and the twenty-four hour excretions in urine and secretions in milk during the first ten days post partum are given in Table I. The average values per day for pantothenic acid intake, secretion in milk, and excretion in urine during all five-day periods of study while the mothers were secreting immature and mature milk are given in Table II.

The average daily pantothenic acid intakes during the two five-day periods of study immediately following delivery ranged from 6.0 to 9.5 mg. (Table I). In general, during the first ten days post partum secretion of the vitamin in milk increased in direct proportion to the increase in volume of milk produced. The influence of nutritional status and physiologic state upon the composition of breast milk is indicated by the high concentrations in the milk of V. S., who ingested multivitamin supplements throughout pregnancy in addition to an excellent diet, and by the low values of C. O., who developed a low-grade infec-

\*Mylase P is a diastase preparation obtainable from Wallerstein Laboratories, New York, N. Y., which is more active than clarase in liberating pantothenate and contains less pantothenate.

TABLE I. TWENTY-FOUR HOUR PANTOTHENIC ACID EXCRETION IN URINE AND SECRETION IN MILK DURING FIRST TEN DAYS POST PARTUM

SUBJECT	INTERVAL POST PARTUM (DAYS)	VOLUMES		PANTOTHENIC ACID	
		MILK (ML.)	URINE (ML.)	MILK (MG.)	URINE (MG.)
L. F.	1	71	2590	0.03	3.31
	2	200	1275	0.20	3.23
	3	733	2120	1.53	3.80
	4	1122	2075	3.33	4.32
	5	1441	1674	3.89	5.76
	6	1501	1718	3.81	5.51
	7	1596	1340	5.36	4.29
	8	1638	1810	5.45	3.89
	9	1676	1371	6.12	3.03
	10	1872	2220	5.80	4.69
V. G.	1	82	2067	0.02	4.62
	2	177	1689	0.22	4.19
	3	819	1775	1.66	4.98
	4	1413	1323	2.87	3.86
	5	1471	1303	3.06	4.77
	6	1782	1148	4.06	4.73
	7	1630	1230	4.14	4.44
	8	1895	1237	4.30	4.60
	9	1828	1104	4.02	4.15
	10	1770	1075	4.04	4.17
V. K.	1	9	3086	0.00	4.01
	2	90	3651	0.13	4.88
	3	484	2596	1.01	4.70
	4	517	2427	1.30	4.70
	5	560	2331	1.57	5.40
	6	663	2249	1.66	5.12
	7	781	3380	2.05	5.23
	8	775	3840	2.06	5.56
	9	794	3115	1.98	5.13
	10	797	3328	2.19	5.83
V. L.	1	30	1757	0.01	4.28
	2	56	2686	0.03	4.19
	3	353	2759	0.72	4.56
	4	794	1823	1.89	4.92
	5	844	1817	2.55	5.41
	6	955	2710	2.69	5.56
	7	1047	2841	3.65	6.12
	8	1098	2066	3.76	5.40
	9	1118	2151	3.39	4.46
	10	1200	1669	3.72	4.16
J. M.	1	35	2182	0.02	4.42
	2	385	1386	0.53	3.97
	3	870	1519	1.80	4.36
	4	1011	1201	2.40	3.39
	5	1121	1283	2.74	4.45
	6	1125	1172	3.03	4.58
	7	1287	1161	3.70	4.73
	8	1136	1520	3.27	5.58
	9	1258	-	3.26	-
	10	1336	1553	3.82	5.66
C. O.	1	16	1864	0.00	4.20
	2	100	939	0.04	2.98
	3	335	1511	0.22	3.33
	4	395	-	0.61	-
	5	725	-	0.96	-
	6	821	-	1.38	-
	7	798	1470	1.37	4.08



TABLE I. (CONT'D)

SUBJECT	INTERVAL POST PARTUM (DAYS)	VOLUMES		PANTOTHENIC ACID	
		MILK (ML.)	URINE (MG.)	MILK (MG.)	URINE (MG.)
	8	950	1127	1.62	4.92
	9	931	1191	1.58	3.88
	10	660	—	0.77	—
V. S.	1	6	1869	—	5.86
	2	92	1874	0.12	5.11
	3	420	2887	1.10	6.62
	4	600	2063	1.55	5.42
	5	697	2065	1.93	5.88
	6	756	1502	2.36	5.29
	7	818	2524	2.54	6.85
	8	837	2237	3.33	7.59
	9	932	1984	3.26	6.68
	10	924	2287	3.81	6.97
M. S.	7	880	1355	2.99	5.51
G. S.	7	1017	1981	2.29	5.20
F. W.	7	953	1032	1.29	4.17

The first day post partum was variable to the extent of differences in the times at which the women delivered. For subjects delivered after 12 M. the first day began the following morning. The first day for C. O. was 22 hours; for L. F., 23.5 hours. V. G. was delivered eight hours before the beginning of the first day post partum; V. K., seventeen hours; V. L., five hours; J. M., sixteen hours; and V. S., seven hours.

tion near the end of the puerperium. Excretion of pantothenic acid in urine per twenty-four hours was relatively constant for each woman but was not related to urine volume.

The average values per day during all five-day periods (Table II) show the wide variation in milk and urine volumes among the subjects and for the same subject at different times. Although the averages for pantothenate in milk during the two five-day periods immediately post partum portray the large increases in the daily values (Table I) during the first five days, it is interesting that there were only slight differences between the average percentages of intake found in the urine of each woman during the two five-day periods. The greatest difference was an increase from 61 per cent during the first five days to 71 per cent of intake excreted in urine during the second five days by V. S. who had received multivitamin supplements throughout pregnancy. From 70 to 93 per cent of the pantothenic acid intakes of six women during the first five days post partum was accounted for in milk and urine. The range values increased to 94 to 131 per cent for the next five days, with an average increase of 31 per cent. Since the concentration of pantothenate in mature human milk<sup>9</sup> is quite consistent, the values per day in Table II correspond generally with milk volumes. Excretion in urine showed no relationship with urine volume. For all periods of secretion of mature milk from 49 to 103 per cent of the intake was accounted for in milk and urine, with an average of 77 per cent.

#### DISCUSSION

The average daily urinary excretions of pantothenate for the various five-day periods are comparable to the average daily figures of 4.94 mg. given by

TABLE II. AVERAGE DAILY PANTOTHENIC ACID INTAKE; EXCRETION IN URINE AND SECRETION IN MILK DURING THIRTY-ONE FIVE-DAY PERIODS

SUBJECT	INTERVAL POST PARTUM (DAYS)	VOLUMES		PANTOTHENIC ACID				
		MILK (ML.)	URINE (ML.)	INTAKE (MG.)	MILK		URINE	
					MG.	% INTAKE	(MG.)	% INTAKE
M. B.	72-76	718	691	6.9	1.17	17	2.20	32
L. F.	1-5	713	2007	8.0	1.86	23	4.08	51
	6-10	1657	1692	7.7	5.31	68	4.28	55
V. G.	1-5	798	1631	6.5	1.56	24	4.18	69
	6-10	1781	1159	6.5	4.11	63	4.42	68
	78-82	818	1281	7.8	2.24	29	4.70	60
	161-165	901	993	8.6	2.16	25	4.72	55
	239-243	681	810	8.0	1.36	17	3.80	48
	302-306	394	1080	7.6	1.05	14	4.74	62
V. K.	1-5	338	2818	6.2	0.80	13	4.74	76
	6-10	762	3182	6.8	1.99	29	5.38	80
	95-99	647	2804	8.6	1.85	21	5.10	59
	144-148	325	1447	8.2	0.98	12	4.50	55
V. L.	1-5	415	2174	8.2	1.04	13	4.67	57
	6-10	1084	2287	9.1	3.44	38	5.14	56
	68-72	789	2849	9.2	2.28	25	3.64	39
	152-156	680	1756	9.4	1.96	21	3.91	42
J. M.	1-5	684	1514	7.9	1.50	19	4.12	52
	6-10	1228	1352	8.9	3.42	38	5.14	58
	75-79	708	2265	9.4	2.13	22	5.12	54
	173-177	268	2003	8.1	0.78	10	4.75	58
C. O.	1-5	354	1438	6.0	0.36	6	—	—
	6-10	832	1263	6.2	1.34	22	—	—
B. S.	85-89	1020	1347	6.8	2.05	30	4.23	62
	204-208	913	1356	7.1	2.09	30	5.18	73
	259-263	676	1219	8.2	1.85	23	5.17	63
G. S.	80-84	899	1078	6.7	2.30	34	3.87	58
V. S.	1-5	363	2152	9.5	0.94	10	5.78	61
	6-10	853	2107	9.4	3.06	33	6.68	71
	70-74	304	1852	6.9	0.88	13	6.17	89
M. S.	58-62	947	939	9.1	2.04	22	4.18	46

Spector and co-workers<sup>25</sup> for normal adult men under "comfortable conditions," but are somewhat higher than the figure 3.5 mg. per day given by Sarett<sup>26</sup> for three normal men on normal diets, or the average values of 3.8, 3.2, and 3.4 mg. found for the normal subjects of Pelczar and Porter,<sup>27</sup> Pearson,<sup>28</sup> and Wright and Wright,<sup>29</sup> respectively. Gardner and associates<sup>10</sup> in a ten-day study, determined the riboflavin, pantothenic acid, and biotin contents of the urine and feces of three healthy young women whose only food other than caramel candy was cow's milk supplemented with a mineral mixture and ascorbic acid. The diet furnished only 6.9 mg. of pantothenic acid daily and the daily urinary excretion of the vitamin averaged 6.0 mg., 87 per cent of the intake. The fecal excretion averaged only 0.41 mg., 6 per cent of intake.

Oldham, Davis, and Roberts,<sup>31</sup> during studies of twelve young women, determined the pantothenic acid content of seventy-two hour collections of food, urine,

and feces from five to nine of the subjects. During consecutive periods of forty-one to fifty-nine days the women received diets which provided average daily intakes of 2.1, 2.1, 3.5, and 4.3 mg. per day, in that order. During these periods the thiamine and riboflavin intakes also were increased from low levels. Three-day composites for each woman were collected during the last week of the study. At the low level of intake, excretion in both urine and feces was quite consistent, averaging 1.6 and 0.8 mg., 79 and 38 per cent of the intake, respectively. With the increased intake, greater variation occurred among individuals and daily excretions in urine and feces rose. However, the average percentage of intake accounted for in urine decreased with greater intake, lowering the total of intake accounted for from an average of 116 per cent during the first two periods to about 94 per cent during the next two study intervals, which may indicate inadequacy of 2.1 mg. of pantothenate per day to supply the metabolic needs of the women.

With the values given in Table II for nursing mothers, the average daily amounts of pantothenate secreted in milk may be subtracted from the corresponding average daily intakes, with the thesis that only the differences are available for use by the mothers' bodies. These net intakes range from 4.94 to 8.56 mg. for the first five days; from 2.39 to 6.34 mg. for the second five days post partum; and from 4.40 to 7.44 mg. during the periods of mature milk production.

From the data for the first ten days post partum it seems evident that the maternal body can store pantothenic acid or that it is retained in the body for more than twenty-four hours during its metabolism. For the first five days post partum the average net intakes of six women exceeded the respective average daily excretions in urine by 0.46 to 2.78 mg.; during the next five days the average daily urinary excretion by four of the women was greater than the average net intakes. Apparently, physiologic levels of pantothenic acid are maintained in both the milk and urine of nursing mothers regardless of current intake if the women's nutritional status with respect to the vitamins is good. Although some workers believe that some pantothenic acid may be available to the body from fecal synthesis, this has not been proved. Since these nursing mothers, receiving a diet providing ample amounts of the vitamin, excreted over 100 per cent of the net intake in urine it seems unlikely that the feces play a significant role in excretion of pantothenic acid intake.

During the periods of mature milk production the average daily secretions in milk and excretions in urine showed no relationship to each other, to urine volumes, or to average daily intakes. Nor was urinary excretion related to milk volume. Although one woman (M. B.) showed an average of only 49 per cent of her intake in urine and milk, from 63 to 103 per cent of intake appeared in urine and milk during the sixteen experimental periods for eight other women. With intakes averaging 6.0 to 9.5 mg. per day, 32 to 89 per cent appeared in the urine, and the variation in urinary excretion occurred in the same individual as well as among the group. It seems doubtful that any great amount of the pantothenic acid was excreted in the feces.

## SUMMARY

Pantothenic acid was determined in five-day composites of food and twenty-four hour collections of the milk and urine of seven multiparas during two five-day periods immediately post partum and of nine nursing mothers during seventeen five-day periods at intervals during the production of mature milk.

The average daily intakes of pantothenic acid ranged from 6.0 to 9.5 milligrams. During the first ten days post partum, daily secretion in milk rose with increasing volumes of milk produced. During the five days immediately following delivery, the intakes of six women were 0.46 to 2.78 mg. in excess of secretion in the milk and excretion in urine; during the next five days the milk and urine of four of the women contained averages of more pantothenic acid than their diets furnished. During the periods of mature milk production, 49 to 103 per cent of intake was found in milk and urine. The urine alone contained 32 to 89 per cent of the average daily intakes during the five-day periods.

## REFERENCES

1. Jukes, Thomas H.: *The Distribution of Pantothenic Acid in Certain Products of Natural Origin*, *J. Nutrition* 21: 193-200, 1941.
2. Nelson, Marjorie M., and Evans, Herbert M.: *Pantothenic Acid Deficiency and Reproduction in the Rat*, *J. Nutrition* 31: 497-507, 1946.
3. Gillis, M. B., Heuser, G. F., and Norris, L. C.: *The Need for Pantothenic Acid and an Unidentified Factor in Reproduction in the Domestic Fowl*, *J. Nutrition* 23: 153-163, 1942.
4. Unna, Klaus, and Richards, Grace V.: *Relationship Between Pantothenic Acid Requirement and Age in the Rat*, *J. Nutrition* 23: 515-553, 1942.
5. Silber, Robert H.: *Studies of Pantothenic Acid Deficiency in Dogs*, *J. Nutrition* 27: 425-433, 1941.
6. Macy, Icie G., Williams, Harold H., Pratt, J. P., and Hamil, Brenton, M.: *Human Milk Studies. XIX. Implications of Breast Feeding and Their Investigation*, *Am. J. Dis. Child.* 70: 135-141, 1945.
7. Kaucher, Mildred, Moyer, Elsie Z., Richards, Allen J., Williams, Harold H., Wertz, Annie Lou, and Macy, Icie G.: *Human Milk Studies. XX. The Diet of Lactating Women and the Collection and Preparation of Food and Human Milk for Analysis*, *Am. J. Dis. Child.* 70: 142-147, 1945.
8. Davies, Velma: *Human Milk Studies. XXI. A Simple Technic for the Manual Expression of Mother's Milk*, *Am. J. Dis. Child.* 70: 148-149, 1945.
9. Coryell, Margaret N., Harris, Mary Ellen, Miller, Solomon, Williams, Harold H., and Macy, Icie G.: *Human Milk Studies. XXII. Nicotinic Acid, Pantothenic Acid and Biotin Contents of Colostrum and Mature Human Milk*, *Am. J. Dis. Child.* 70: 150-161, 1945.
10. Roderuck, Charlotte E., Williams, Harold, H., and Macy, Icie G.: *Human Milk Studies. XXIII. Free and Total Thiamine Contents of Colostrum and Mature Human Milk*, *Am. J. Dis. Child.* 70: 162-170, 1945.
11. Roderuck, Charlotte E., Coryell, Margaret N., Williams, Harold H., and Macy, Icie G.: *Human Milk Studies. XXIV. Free and Total Riboflavin Contents of Colostrum and Mature Human Milk*, *Am. J. Dis. Child.* 70: 171-175, 1945.
12. Munks, Bertha, Robinson, Abner, Williams, Harold H., and Macy, Icie G.: *Human Milk Studies. XXV. Ascorbic Acid and Dehydroascorbic Acid in Colostrum and Mature Human Milk*, *Am. J. Dis. Child.* 70: 176-181, 1945.
13. Leshner, Marjorie, Brody, James K., Williams, Harold H., and Macy, Icie G.: *Human Milk Studies. XXVI. Vitamin A and Carotenoid Contents of Colostrum and Mature Human Milk*, *Am. J. Dis. Child.* 70: 182-192, 1945.
14. Pratt, J. P., Kaucher, Mildred, Richards, Allen J., Williams, Harold H., and Macy, Icie G.: *Composition of the Human Placenta. I. Proximate Composition*, *Am. J. Obst. & Gynec.* 52: 402-408, 1946.
15. Pratt, J. P., Kaucher, Mildred, Moyer, Elsie Z., Richards, Allen J., and Williams, Harold H.: *Composition of the Human Placenta. II. Lipid Content*, *Am. J. Obst. & Gynec.* 52: 665-668, 1946.
16. Pratt, J. P., Roderuck, Charlotte, Coryell, Margaret, and Macy, Icie G.: *Composition of the Human Placenta. III. Vitamin Content*, *Am. J. Obst. & Gynec.* 52: 783-787, 1946.

17. Munks, Bertha, Kaucher, Mildred, Moyer, Elsie Z., Harris, Mary Ellen, and Macy, Icie G.: Metabolism of Women During the Reproductive Cycle. XI. Vitamin C in Diets, Breast Milk, Blood and Urine of Nursing Mothers, *J. Nutrition* 33: 601-619, 1947.
18. Leshner, Marjorie, Brody, James K., Williams, Harold H., and Macy, Icie G.: Metabolism of Women During the Reproductive Cycle. X. The Utilization of Vitamin A During Lactation, *J. Am. Dietet. A.* 23: 211-217, 1947.
19. Roderuck, Charlotte, Williams, Harold H., and Macy, Icie G.: Metabolism of Women During the Reproductive Cycle. VIII. The Utilization of Thiamine During Lactation, *J. Nutrition* 32: 249-265, 1946.
20. Roderuck, Charlotte, Coryell, Margaret N., Williams, Harold H., and Macy, Icie G.: Metabolism of Women During the Reproductive Cycle. IX. The Utilization of Riboflavin During Lactation, *J. Nutrition* 32: 267-283, 1946.
21. Coryell, Margaret, Roderuck, Charlotte, Harris, Mary Ellen, Miller, Sol, Rutledge, Marjorie, and Macy, Icie G.: Metabolism of Women During the Reproductive Cycle. XIII. The Utilization of Niacin During Lactation, *J. Nutrition* 34: 219-231, 1947.
22. Coryell, Margaret, Harris, Mary Ellen, Miller, Sol, Rutledge, Marjorie, Williams, Harold H., and Macy, Icie G.: Metabolism of Women During the Reproductive Cycle. XV. The Utilization of Biotin During Lactation, *J. LAB. & CLIN. MED.* 32: 1462, 1947.
23. Pratt, J. P., Munks, Bertha, and Macy, Icie G.: Metabolism of Women During the Reproductive Cycle. XII. The Effect of Multiple Vitamin Supplements on the Vitamin C Utilization of Healthy Nursing Mothers, *J. Nutrition* 33: 621-632, 1947.
24. Ives, Margaret, and Strong, F. M.: Preparation of Samples for the Microbiological Assay of Pantothenic Acid, *Arch. Biochem.* 9: 251-258, 1946.
25. Spector, H., Hamilton, T. S., and Mitchell, H. H.: The Effect of Pantothenic Acid Dosage and Environmental Temperature and Humidity Upon the Dermal and Renal Excretion of Pantothenic Acid, *J. Biol. Chem.* 161: 145-152, 1945.
26. Sarett, Herbert P.: Metabolism of Pantothenic Acid and Its Lactone Moiety in Man, *J. Biol. Chem.* 159: 321-325, 1945.
27. Pelczar, Michael J., Jr., and Porter, J. R.: Determination of Pantothenic Acid in Normal Blood and Urine by Microbiological Technic, *Proc. Soc. Exper. Biol. & Med.* 47: 3-7, 1941.
28. Pearson, Paul B.: Studies on the Metabolism of Pantothenic Acid in Men and Rabbits, *Am. J. Physiol.* 135: 69-76, 1941.
29. Wright, Lemuel D., and Wright, Ernestine Q.: Urinary Excretion of Pantothenic Acid by Normal Individuals, *Proc. Soc. Exper. Biol. & Med.* 49: 80-81, 1942.
30. Gardner, Josephine, Neal, A. Leslie, Peterson, William H., and Parsons, Helen T.: Biotin, Pantothenic Acid, and Riboflavin Balances of Young Women on a Milk Diet, *J. Am. Dietet. A.* 19: 683-684, 1943.
31. Oldham, H. G., Davis, M. V., and Roberts, L. J.: Thiamine Excretions and Blood Levels of Young Women on Diets Containing Varying Levels of the B Vitamins With Some Observations on Niacin and Pantothenic Acid, *J. Nutrition* 32: 163-180, 1946.

# METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE

## XV. THE UTILIZATION OF BIOTIN DURING LACTATION

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SPECIFIC biologic effects of biotin and of the lack of it have been demonstrated in various animals and plants, but there is little indication of its basic function in metabolism. Nevertheless, universal occurrence of this vitamin in living cells, its great biologic potency per unit weight, and the knowledge that it plays an extremely important role in the health and growth of animals and plants suggest an equally important function of biotin in the human being. That biotin may be a component of the enzyme systems concerned with the metabolism of pyruvate or lactate has been indicated by the *in vitro* tissue experiments of Pilgrim and co-workers<sup>1</sup> and Summerson and associates.<sup>2</sup> That it plays a role as a coenzyme in the transfer of carbon dioxide has also been suggested.<sup>3</sup> In 1941, West and Woglum<sup>4</sup> noted, in rats, that some embryo tissues (skin, muscle, lung, and brain) contained two to thirteen times the concentration of biotin found in corresponding adult tissues. More recently, rat experiments by Kennedy and Palmer<sup>5</sup> have indicated the necessity of biotin for successful gestation, for the birth of viable young, and probably for successful lactation. However, the biotin content of the placenta is singularly low in comparison with the concentration in other organs.<sup>6</sup>

During a comprehensive investigation of the utilization of nutriment from food by normal lactating women, biotin was determined in their food, milk, and urine during the first ten days post partum and during five-day periods at intervals during mature milk production. References are given in a preceding paper<sup>7</sup> to publications containing the details of the selection of subjects and the organization of the study; the dietary; the method of manually expressing the milk; the concentration of niacin, pantothenic acid, biotin, thiamine, riboflavin, ascorbic acid, vitamin A, and carotene in human milk; the proximate composition, lipid and vitamin contents of human placentas; and the utilization of ascorbic acid, vitamin A and carotenoids, thiamine, riboflavin and niacin by the nursing mothers.

### PROCEDURE

The subjects of the study were multiparas selected during the first trimester of pregnancy on the basis of physical and laboratory examinations and records

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of having successfully nursed their other children. During all periods of study the women received diets comparable qualitatively but varying in the quantity of all foods, except milk, according to appetite. Different menus were served each day of the five-day study periods and repeated during each period. Aliquot portions of each food, as eaten, were weighed into a composite which was accumulated for each five-day period of study. Both breasts were completely emptied by manual expression at the usual nursing intervals and samples were taken for analysis from the complete twenty-four hour collection of milk. The entire excretion of urine during each twenty-four hours also was obtained for analysis.

Seven women were studied throughout the first ten days post partum. One-day values were obtained for five other women on the seventh, eighth, or ninth days following delivery. Five of the seven women and four others were studied during seventeen five-day periods while they were producing mature milk.

#### METHODS

The general microbiologic procedure employing *Lactobacillus arabinosus* has been described,<sup>8</sup> including the preparation of food<sup>9</sup> and milk samples for assay.<sup>8</sup>

Numerous control experiments on the food composites showed that maximum values for biotin, representing the sum of the free and bound biotin contents, were obtained by autoclaving the food aliquots in 2 N H<sub>2</sub>SO<sub>4</sub> for three hours at 15 pounds pressure. Stronger or different acids or longer periods of heating did not increase the values obtained. Neutralization with NaOH gave values not significantly different from those obtained after neutralization with Ba(OH)<sub>2</sub> and subsequent removal of the BaSO<sub>4</sub> on filtering. Since the presence of the sulfate ions did not effect the growth of the organism in the assay, NaOH was employed. After neutralization, the autoclaved 5 ml. aliquots were diluted to 500 ml. and filtered through Whatman No. 42 filter paper. The filtrate was diluted further to a concentration suitable for assay, usually 1:200.

The so-called free or loosely bound biotin in the food samples was determined by autoclaving 5 ml. aliquots of the homogenized material with 200 ml. of 0.1 N HCl. When cool, the mixture was brought to pH 5.0 with NaOH, diluted to 250 ml., filtered, and the filtrate diluted to 1:100 or 1:125.

The urine was filtered if necessary and the acetic acid, added as a preservative, neutralized by adjusting the pH to 6.5 to 7.0 before diluting to a concentration of 1:100 to 1:250 for assay.

No attempt was made to differentiate between biotin and its oxygen analogue *dl*-oxybiotin.<sup>10</sup> This vitamer has been shown to have about one-half the activity of *d*-biotin for *L. arabinosus*<sup>11</sup> and perhaps one-tenth of the activity of *d*-biotin for rats.<sup>12</sup> In the only available assays of foods, *dl*-oxybiotin was not found in the materials examined, namely organ and muscle tissues and yeast.<sup>13</sup> Oppel<sup>14</sup> has reported the presence in dog, rabbit, rat, and human urine of a nonavidin-combining biotin, presumably a vitamer.<sup>14</sup> This biotin-like substance was active for his test organism, *Saccharomyces cerevisiae*.<sup>14</sup> It is evidently not oxybiotin, however, since the latter combines with avidin as well as biotin.

## RESULTS AND DISCUSSION

The milk and urine volumes per twenty-four hours and the amounts of biotin in the milk and urine of each subject during the first ten days post partum are recorded in Table I.

TABLE I. TWENTY-FOUR HOUR BIOTIN EXCRETION IN URINE AND SECRETION IN MILK DURING FIRST TEN DAYS POST PARTUM

SUBJECT	INTERVAL POST PARTUM (DAYS)	VOLUME		BIOTIN	
		MILK (ML.)	URINE (ML.)	MILK ( $\mu$ G)	URINE ( $\mu$ G)
L. F.	1	71	2590	0.03	18
	2	200	1275	0.05	3
	3	733	2120	0.00	5
	4	1122	2075	0.00	6
	5	1411	1674	0.89	9
	6	1501	1718	2.13	7
	7	1596	1340	0.51	10
	8	1638	1810	0.24	12
	9	1676	1371	0.44	14
	10	1872	2220	1.65	25
V. G.	1	82	2067	0.04	6
	2	177	1689	0.08	9
	3	849	1775	0.36	13
	4	1413	1323	—	13
	5	1471	1303	1.25	43
	6	1782	1148	2.99	33
	7	1630	1230	4.22	32
	8	1895	1237	10.50	37
	9	1828	1104	13.82	31
	10	1770	1075	25.17	59
V. K.	1	9	3086	—	5
	2	90	3651	<0.04	4
	3	484	2596	<0.19	5
	4	547	2427	<0.27	5
	5	560	2331	0.30	14
	6	663	2249	0.52	14
	7	781	3380	0.69	25
	8	775	3840	0.96	20
	9	794	3115	1.31	14
	10	797	3328	3.60	40
V. L.	1	30	1757	—	4
	2	56	2686	—	4
	3	353	2759	—	3
	4	794	1823	—	6
	5	844	1847	—	34
	6	955	2710	0.52	12
	7	1047	2841	0.51	15
	8	1098	2066	0.86	15
	9	1118	2151	1.92	26
	10	1200	1669	12.72	55
J. M.	1	35	2182	<0.02	11
	2	385	1386	<0.19	—
	3	870	1519	<0.87	11
	4	1011	1201	<1.01	16
	5	1121	1283	3.46	68
	6	1125	1172	3.06	30
	7	1287	1161	4.44	29
	8	1136	1520	5.79	38
	9	1258	—	8.25	—
	10	1336	1533	23.65	88



TABLE I. (CONT'D)

SUBJECT	INTERVAL POST PARTUM (DAYS)	VOLUME		BIOTIN	
		MILK (ML.)	URINE (ML.)	MILK ( $\mu$ G)	URINE ( $\mu$ G)
C. O.	1	16	1864	0.00	18
	2	100	939	0.03	4
	3	335	1511	0.10	5
	4	595	—	—	—
	5	725	—	0.44	—
	6	821	—	0.90	—
	7	798	1470	0.72	6
	8	950	1127	0.76	10
	9	931	1191	0.84	12
	10	660	—	1.03	—
V. S.	1	6	1869	—	9
	2	92	1874	—	4
	3	420	2887	—	10
	4	600	2063	0.35	13
	5	697	2065	0.84	39
	6	756	1502	0.98	23
	7	818	2524	1.67	34
	8	837	2237	3.57	40
	9	932	1984	2.63	51
	10	924	2287	23.65	100
M. B.	9	1189	1035	7.49	28
E. L.	8	688	677	5.50	7
M. S.	7	880	1355	0.97	4
G. S.	7	1017	1981	0.64	14
F. W.	7	953	1032	0.70	2

The first day post partum was variable to the extent of differences in the time at which the women delivered. For subjects delivered after 12 M. the first day began the following morning. The first day for C. O. was 22 hours; for L. F., 23.5 hours. V. G. was delivered eight hours before the beginning of the first day of study; V. K., seventeen hours; V. L., five hours; J. M., sixteen hours; and V. S., seven hours.

The values for biotin in milk for the first four or five days following parturition were too low to be reliable. During the second five days both concentration and daily secretion of biotin in milk varied widely among the seven women, ranging from 0.24  $\mu$ g per day (0.015  $\mu$ g per 100 ml.) to 25.17  $\mu$ g per day (2.56  $\mu$ g per 100 ml.). Some of the values for the fifth and tenth days show abrupt increases, probably owing to the inclusion of liver, which is rich in biotin, in the menus for the fifth day of each period. Although the subjects show small increases in the biotin content of the milk by the fifth day post partum, they are not nearly so marked as on the tenth day.

Unlike niacin<sup>9</sup> and pantothenic acid,<sup>7</sup> biotin excretion in the urine is quite low during the first four days following parturition, increasing several times during the second five days. Increases in urinary excretion on the fifth and tenth days are similar to increased secretions in milk on the days in which liver was included in the diets. Similar changes in the riboflavin content of the milk and urine were noted on those days.<sup>15</sup> It is noteworthy that the milk of L. F. contained very small amounts of biotin during the first ten days post partum.

TABLE II. AVERAGE DAILY BIOTIN INTAKES, EXCRETION IN URINE AND SECRETION IN MILK DURING TWENTY-NINE FIVE-DAY PERIODS

SUBJECT	INTERVAL POST PARTUM (DAYS)	VOLUMES		BIOTIN					
				INTAKE		MILK		URINE	
		MILK (ML.)	URINE (ML.)	FREE ( $\mu$ G)	TOTAL ( $\mu$ G)	( $\mu$ G)	% IN- TAKE*	( $\mu$ G)	% IN- TAKE*
M. B.	72-76	718	691	42	52	0.9	2	8	15
L. F.	1-5	713	2007	58	88	0.2	0	8	9
	6-10	1657	1692	56	102	1.0	1	14	14
V. G.	1-5	798	1631	63	99	0.4	0	17	17
	6-10	1781	1159	53	67	11.3	17	38	57
	78-82	848	1281	61	88	9.6	11	43	49
	161-165	901	993	56	73	5.1	7	37	50
	239-243	681	810	70	96	4.3	4	40	42
	302-306	394	1080	55	91	4.0	4	40	44
V. K.	1-5	338	2818	47	64	<0.2	0	6	9
	6-10	762	3182	56	66	1.4	2	22	33
	95-99	617	2804	61	78	8.4†	11	52	67
	144-148	325	1417	43	65	1.7	3	24	37
V. L.	1-5	415	2174	65	117	—	—	10	8
	6-10	1081	2287	65	113	3.3	3	25	22
	68-72	789	2849	46	71	5.5	8	29	41
	152-156	680	1756	62	98	6.3	6	39	40
J. M.	1-5	684	1514	64	109	<1.1	1	26†	24
	6-10	1228	1352	60	95	9.0	9	46†	48
	75-79	708	2265	52	83	5.1	6	37	45
	173-177	268	2003	70	117	1.8	2	38	32
C. O.	1-5	354	1438	43	52	0.1	0	—	—
	6-10	832	1263	54	82	0.8	1	—	—
B. S.	85-89	1020	1317	44	68	5.6	8	25	37
	204-208	913	1356	58	80	10.8	14	51	64
	259-263	676	1219	58	75	8.3	11	52	70
M. S.	58-62	947	939	67	83	9.8	12	46	55
G. S.	80-84	899	1078	45	73	6.3	9	29	40
V. S.	1-5	363	2152	61	109	—	—	15	14
	6-10	853	2107	65	93	6.5	7	49	52
	70-74	304	1852	58	82	3.0	4	54	66

\*Percentage of intake of total biotin.

†Averages of values for four days.

although in urine she excreted amounts comparable to those of other subjects and by the tenth day was secreting a large volume of milk. That the concentration of biotin in the milk was not lowered by a diluting effect of the high volume secreted is indicated by the values for V. G. who with similar high volume maintained a high concentration of biotin in her milk.

For all five-day periods of study the average daily milk and urine volumes are given in Table II, with the average daily biotin secretions in milk and excretions in urine. For biotin intake both free or loosely bound biotin, as determined after hydrolysis with weak acid for a comparatively short time, and total biotin, the sum of the loosely and firmly bound biotin as determined after hydrolysis with strong acid for long periods of time, are given. It is generally

assumed that the total biotin more nearly represents that available to the animal organism. The biotin in the milk and urine is apparently in the free state, since the values do not increase after hydrolysis with strong acid for long periods of time.

During the periods of mature milk production averages of 2 to 14 per cent of the total biotin intakes were found in the milk secreted during the five-day periods. From 15 to 70 per cent of intake was found in the urine. The diets, which have been described in detail,<sup>16</sup> by analysis provided an average daily intake of 55.8  $\mu\text{g}$  of free biotin and 80.8  $\mu\text{g}$  of total biotin. These values are somewhat higher than those reported for average diets by other investigators. Oppel<sup>17</sup> found daily averages of 30 to 37  $\mu\text{g}$  of total biotin in average diets. Gardner and co-workers<sup>18</sup> reported an average of 33  $\mu\text{g}$  in a moderate-biotin diet which contained no liver or eggs and provided fewer calories and lesser amounts of other vitamins. For diets containing similar amounts of energy and other vitamins, Denko and associates<sup>19</sup> found 37 to 54  $\mu\text{g}$  of biotin, with an average of 44  $\mu\text{g}$  daily. These workers, however, used an enzyme method of extraction<sup>20</sup> which in our hands gave the loosely-bound values for biotin in food composites.

The biotin in milk represented a small percentage of the total biotin in the food during the mature milk periods, ranging from 2 to 14 per cent, in comparison with 10 to 34 per cent for pantothenic acid,<sup>7</sup> 2 to 15 per cent for thiamine,<sup>21</sup> and 3 to 15 per cent for riboflavin.<sup>15</sup> The average figure for biotin excretion in urine during the periods of mature milk secretion was 47 per cent. With the difficulties of complete extraction of biotin from a food composite and the uncertainty as to whether the values obtained accurately represent the amounts available to the subjects who consume the food, it is not surprising that we found little relationship between average intakes within a narrow range, secretion in milk, and excretion in urine by nursing mothers. Together, milk and urine accounted for 54 per cent of the biotin ingested in the food. With daily intakes of 30 to 37  $\mu\text{g}$ , Oppel<sup>17</sup> found average excretion values of 29 to 54  $\mu\text{g}$  for normal men, about 85 to 159 per cent of the intakes. Denko and co-workers,<sup>19</sup> working with normal young men, found in urine an average of 32  $\mu\text{g}$  or 73 per cent of the average intake of 44 micrograms. Gardner and associates<sup>18</sup> reported that ten normal young women excreted averages of 29, 30, and 167  $\mu\text{g}$ , or 322, 91, and 98 per cent of intakes of 9, 33, and 171  $\mu\text{g}$  per day, respectively. In an earlier study of young women consuming a mineralized milk diet containing 115  $\mu\text{g}$  of biotin daily, Gardner and co-workers<sup>22</sup> found an average urinary excretion of 50  $\mu\text{g}$ , 43 per cent of the intake.

Fecal biotin may well be a factor in biotin metabolism, since it has been shown that intestinal synthesis is an important source of biotin in many experimental animals. While fecal analyses do not answer the question as to whether the biotin is unabsorbed intake or the product of intestinal synthesis, or to what extent it has been available to the body, Gardner and co-workers<sup>22</sup> found 73  $\mu\text{g}$ , 64 per cent of the intake, in the feces. In another study<sup>19</sup> subjects with intakes of 9, 33, and 171  $\mu\text{g}$  per day excreted 55, 78, and 100  $\mu\text{g}$  in the feces, 611, 236, and 58 per cent, respectively, of the intakes. Oppel<sup>17</sup> found that his subjects on average general diets excreted 86 to 191  $\mu\text{g}$  of fecal biotin per day

Denko and associates<sup>10</sup> reported an average fecal biotin excretion of 133  $\mu\text{g}$  daily, 302 per cent of the intake. These investigators noted that 60 to 100 per cent of the vitamins found in the feces were water-extractable.

The results of experimental work with animals and human beings have established the synthesis of biotin by intestinal bacteria. Moreover, the findings that with low intake the urinary excretion of biotin may be much larger than intake and that a large portion of the fecal vitamin is water-extractable indicate availability of fecal biotin to the body. If further work substantiates this, the wide range of response by individuals in a group might be explained by variation in fecal synthesis.

#### SUMMARY

Biotin was determined in five-day composites of food and twenty-four hour collections of the milk and urine of seven multiparas during two five-day periods immediately post partum and of nine nursing mothers during seventeen five-day periods at intervals during the production of mature milk.

The values for biotin in milk for the first four days following parturition were too low to be reliable. During the second five days both concentration and daily secretion of biotin in the milk varied widely among the seven women, ranging from 0.24  $\mu\text{g}$  per day (0.015  $\mu\text{g}$  per 100 ml.) to 25.17  $\mu\text{g}$  per day (2.56  $\mu\text{g}$  per 100 ml.). During the periods of mature milk production averages of 2 to 14 per cent of the total biotin intakes (0.9 to 10.8  $\mu\text{g}$  per day) were found in the milk secreted during the five-day periods. From 15 to 70 per cent of the intakes (8 to 54  $\mu\text{g}$  per day) was found in the urine.

#### REFERENCES

1. Pilgrim, Francis J., Axelrod, A. E., and Elvehjem, C. A.: The Metabolism of Pyruvate by Liver From Pantothenic- and Biotin-Deficient Rats, *J. Biol. Chem.* 145: 237-240, 1942.
2. Summerson, William H., Lee, Johanna M., and Partridge, Chester W. H.: The Effect of Biotin on the Metabolism of Liver Slices From Biotin-Deficient Rats, *Science* 100: 250-251, 1944.
3. Burk, Dean, and Winzler, Richard J.: Heat-Labile, Avidin-Uncombinable, Species-Specific and Other Vitamins of Biotin, *Science* 97: 57-60, 1943.
4. West, Philip M., and Woglum, William H.: The Biotin Content of Tumors and Other Tissues, *Science* 93: 525-527, 1941.
5. Kennedy, Cornelia, and Palmer, Leroy S.: Biotin Deficiency in Relation to Reproduction and Lactation, *Arch. Biochem.* 7: 9-13, 1945.
6. Pratt, J. P., Roderuck, Charlotte, Coryell, Margaret, and Macy, Icie G.: Composition of the Human Placenta. III. Vitamin Content, *Am. J. Obst. & Gynec.* 52: 783-787, 1946.
7. Coryell, Margaret N., Miller, Sol, Williams, Harold H., and Macy, Icie G.: Metabolism of Women During the Reproductive Cycle. XIV. The Utilization of Pantothenic Acid During Lactation, *J. LAB. & CLIN. MED.* 32: 1454, 1947.
8. Coryell, Margaret N., Harris, Mary Ellen, Miller, Solomon, Williams, Harold H., and Macy, Icie G.: Human Milk Studies. XXII. Nicotinic Acid, Pantothenic Acid and Biotin Contents of Colostrum and Mature Human Milk, *Am. J. Dis. Child.* 70: 150-161, 1945.
9. Coryell, Margaret, Roderuck, Charlotte, Harris, Mary Ellen, Miller, Sol, Rutledge, Marjorie, Williams, Harold H., and Macy, Icie G.: Metabolism of Women During the Reproductive Cycle. XIII. The Utilization of Niacin During Lactation, *J. Nutrition* 34: 219-231, 1947.
10. Hofmann, Klaus, and Winnick, Theodore: The Determination of Oxybiotin in the Presence of Biotin, *J. Biol. Chem.* 160: 449-453, 1945.
11. Rubin, S. H., Flower, D., Rosen, F., and Dreker, L.: The Biological Activity of O-Heterobiotin, *Arch. Biochem.* 8: 79-90, 1945.

12. Hofmann, Klaus, McCoy, Richard H., Felton, Jean R., Axelrod, A. E., and Pilgrim, Francis J.: The Biological Activity of Oxybiotin for the Rat and the Chick, *Arch. Biochem.* 7: 393-394, 1945.
13. Luckey, T. D., Moore, P. R., and Elvehjem, C. A.: A Differential Microbiological Assay for O-Heterobiotin, *Proc. Soc. Exper. Biol. & Med.* 61: 97-100, 1945.
14. Oppel, Theodore W.: Studies of Biotin Metabolism in Man. III. The Excretion of Two Biotin-like Substances in Urine, *Am. J. M. Sc.* 204: 869-875, 1942.
15. Roderuck, Charlotte, Coryell, Margaret N., Williams, Harold H., and Macy, Icie G.: Metabolism of Women During the Reproductive Cycle. IX. The Utilization of Riboflavin During Lactation, *J. Nutrition* 32: 267-283, 1946.
16. Kaucher, Mildred, Moyer, Elsie Z., Richards, Allen J., Williams, Harold H., Wertz, Annie Lou, and Macy, Icie G.: Human Milk Studies. XX. The Diet of Lactating Women and the Collection and Preparation of Food and Human Milk for Analysis, *Am. J. Dis. Child.* 70: 142-147, 1945.
17. Oppel, Theodore W.: Studies of Biotin Metabolism in Man. II. The Relationship Between the Biotin Content of the Diet and Its Output in the Urine and Feces, *Am. J. M. Sc.* 204: 863-869, 1942.
18. Gardner, Josephine, Parsons, Helen T., and Peterson, W. H.: Human Biotin Metabolism on Various Levels of Biotin Intake, *Arch. Biochem.* 8: 339-348, 1945.
19. Denko, Charles W., Grundy, Walton E., Porter, John W., Berryman, George H., Friedemann, Theodore E., and Youmans, John B.: The Excretion of B-Complex Vitamins in the Urine and Feces of Seven Normal Adults, *Arch. Biochem.* 10: 33-40, 1946.
20. Cheldelin, Vernon H., Eppright, Margaret A., Snell, Esmond E., and Guirard, Beverly M.: Enzymatic Liberation of B Vitamins From Plant and Animal Tissues, University of Texas Publication No. 4237, 15-36, 1942.
21. Roderuck, Charlotte, Williams, Harold H., and Macy, Icie G.: Metabolism of Women During the Reproductive Cycle. VIII. The Utilization of Thiamine During Lactation, *J. Nutrition* 32: 249-265, 1946.
22. Gardner, J., Neal, A. L., Peterson, W. H., and Parsons, H. T.: Biotin, Pantothenic Acid, and Riboflavin Balances of Young Women on a Milk Diet, *J. Am. Dietet. A.* 19: 683-684, 1943.

## A NUTRITION SURVEY OF VIENNESE CIVILIANS UNDER UNITED STATES OCCUPATION, 1945

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THE responsibility for providing in United States occupied Germany and Austria an equitable distribution of available food supplies fell to the Army at the end of World War II. In order to meet this responsibility with the most facts, nutrition surveys were planned well in advance and begun in each area as it was occupied.<sup>1</sup> Teams were organized, each composed of a clinical nutritionist, a nutrition officer (Sanitary Corps) experienced in general matters of food and nutrition and in taking dietary histories, and a laboratory technician. We formed one such team and report here the results of a survey of the nutritional status of the civilian population of Vienna in the six bezirks occupied by United States forces. The survey was conducted from August 27 until September 15, 1945. It was on September 1 that the United States Army officially took over the administration of the area assigned to it. Because of this the dietary survey is divided into two sections; the first including bezirks 7, 8, and 9, which adjoin the inner section of the city, and the second, bezirks 17, 18, and 19, which were in the outlying districts. The first part of the survey was made during the Russian occupation of the entire city and the second after these six bezirks were administered by the American Army. Pyke<sup>2</sup> has reported a similar survey for the British area of Vienna in September, 1945, and the data are quite comparable to those reported here. Following World War I nutrition surveys in Vienna were made under the direction of the American Relief Administration.<sup>||3, 4</sup>

Consideration will not be given to the previous food intake of the Viennese for two reasons: first, the survey was intended to determine only the food intake at the time the survey was made; and second, reliable figures of food intake during the war were not available. The observations are therefore confined to food intake during the survey itself. It is well known, however, that food was scarce and strictly rationed in Vienna during the war. When the Russian army occupied the city they found that the retreating Germans had left almost no food supplies so that from the end of April until early in June almost no rations at all were issued. During July and August the issue amounted to about 800 calories per day.<sup>2</sup> In general, the Viennese lost weight on rationed food during the war. Thus the Russians, and soon the other members of the four powers,

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found an undernourished population in Vienna, with almost no food available to ration and with grossly inadequate transportation to bring more food. Further, there was an acute shortage of fuel and housing that complicated immensely the public health problems of the city.

#### MATERIALS AND METHODS

*Food Ration Classification.*—At the time of the survey the consumers were divided, according to the Russian method of classification, into five food ration categories: "Heavy Workers; Workers; Employees; Children Under Twelve Years of Age; Others." The "heavy workers" included not only those doing heavy manual labor, but also certain professional people such as physicians, opera singers, and orchestra players. The category "employees" was made up chiefly of white collar workers, and that of "others" of all those not in one of the other categories; that is, housewives, disabled persons, and those too old or otherwise unable to work. The estimated number of individuals falling into each category is shown in the first two columns of Table I.

TABLE I

FOOD RATION CLASSIFICATION	APPROXIMATE POPULATION	TOTAL NUMBER STUDIED	MALE	FEMALE
Heavy workers	10,400	115	105	10
Workers	54,500	540	316	224
Employees	29,600	257	93	164
Children under 14	32,400	548	240	308
Others	138,000	865	86	779
Total	264,900	2,325	840	1,485

*Composition of the Population of the Zone of United States Occupation.*—The United States Vienna Area Command took over the administration of bezirks 7, 8, 9, 17, 18, and 19 on September 1, 1945. Accurate population figures for this area were difficult to obtain, but the closest approximation was about 260,000. The inhabitants of this area varied from the poorest in the city to some of the most wealthy in the suburban districts of Heiligenstadt and Grinzing. Many professional men and women were included as well as workers. The industries located in the area were mostly small.

*Selection of the Sample.*—With the cooperation of the Public Health Division of the United States Vienna Area Command, the Viennese public health officials and police located central sites for the examination and called in individuals and families to represent as nearly as possible 1 per cent of the total number of each of the four food ration categories. Table I shows that, with the exception of the category "others," roughly 1 per cent was examined in each category. The number of children was large. In all dealings with the civilian officials an effort was made not to mention that the survey was concerned with nutrition. The survey was organized, they were told, in an effort to understand the general level of health of the people of Vienna under the administration of United States forces. It was usually possible to make sure that those asked to come did so and that those ill at home were noted. It was impossible to visit those not appearing because of illness, but the number of such was small. Indeed, a few of those who came should have been in bed at home. Great care

was taken to insure that no greater proportion of ill and undernourished individuals was examined than was present in the population. It was not always possible to be sure of this but we believe that with the method of selection used this error was largely avoided. Bezirks 7, 8, and 9 included a large proportion of the poor of Vienna, those likely to suffer first. However, the findings were not strikingly different in the other three bezirks in which the people were better able to afford food purchased in the black market. It was well recognized that food was available in the black market in Vienna at this time, but the quantity was so small that it could, and apparently did, make but little difference in the nutritional status of those able to afford it.

In order to include a sufficient number of heavy workers and workers, three factories were investigated: an optical works, a clothing factory, and a paper factory.

#### *Procedure of the Examination.*—

*Physical Examination:* Each individual removed his clothing except for underclothes and stockings, was weighed (kilograms) and the height measured (centimeters). The age, sex, height, weight, and food ration category were entered beside the individual's serial number, as were the findings in the clinical examination. Table III shows the items on physical examination which were specifically sought. In addition, inquiries were made concerning menses (in women between 15 and 44 years of age), weakness, and syncope. The following physical abnormalities were sought but so rarely seen that they were not listed in the table: purpura, loss of vibratory sense in the great toes, muscular tenderness, absence of knee jerks, bossing of epiphyses, beading of ribs, and cranial bossing.

*Laboratory Examination:* About one of every three of the individuals examined was sent to the laboratory technician and his aides who determined blood hemoglobin and serum proteins by the specific gravity method<sup>2</sup> included in the mobile field laboratory.

#### *Dietary Survey:*

1. Individual dietary history: Slightly over one-third of the individuals reporting for the examination were questioned as to their food intake during the previous twenty-four hours. Models and drawings of food as well as plates and cups were used as aids. The estimated food consumption was calculated by using the composite of the daily results for each class of consumer. In general, the most reliable histories were given by the housewives who prepared the meals for the entire family. In the two factories studied the food served at work for lunch was investigated and the workers themselves were questioned. Reassurance was given that the examiner had no interest in the source of the food (for example, the black market) but only in its quantity. The frequency with which foods appeared which were obtainable only by illicit means was small, corresponding to the estimated size of black market dealings.

2. Rationing and supply of food in Vienna: The food rationing was administered by civilian food authorities and was well organized. Thus it was possible to obtain the ration for each food without any difficulty.



A good insight into the food supplies and the problems of procurement and distribution were obtained by one of us (H. L. W.) who represented the United States in four power conferences on food at the Russian commissariat. It was thus possible to interpret the clinical findings and to some extent to estimate the future nutritional status of the population.

## RESULTS

*Clinical Examination.*—

*Physical Measurements:* The heights and weights were averaged for the several groups formed from the different age, sex, and food ration categories. These were compared with standards for the United States.<sup>6, 7</sup> It must be understood clearly that these standards are about 5 kilograms higher than the weight of adult Austrians on a prewar status. Hereafter, the United States standard used will be referred to as the U. S. standard.

Children, taking the 5-, 10-, and 15-year-old groups as examples, averaged not far below the U. S. standard (Table II). This, however, was not true for

TABLE II. AVERAGE HEIGHT AND WEIGHT OF CHILDREN AGES 5, 10, AND 15 YEARS. WEIGHT COMPARED TO U. S. STANDARDS<sup>6</sup>; BOTH HEIGHT AND WEIGHT COMPARED TO SIMILAR MEASUREMENTS, 1920-21<sup>3, 4</sup>

AGE	SEX	HEIGHT (CM.)		WEIGHT (KG.)		
		VIENNA		VIENNA		UNITED STATES
		1920-1921	1945	1920-1921	1945	
5	Male	--	103	--	16	17
	Female	--	106	--	16	17
10	Male	--	134	27.6	29	30
	Female	--	135	27.7	26	31
15	Male	151	161	42	47	50
	Female	151	156	44	47	48

TABLE III. SYMPTOMS AND SIGNS SUGGESTIVE OF SPECIFIC DEFICIENCIES EXPRESSED IN PERCENTAGE OF EACH GROUP EXAMINED

	CHILDREN BELOW 14 YEARS		HEAVY WORKERS		WORKERS		EMPLOYEES		OTHERS	
	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE
Poor appearance	7	5	16	0	11	7	17	2	24	7
Thyroid enlargement	38	35	41	40	22	44	33	42	28	36
Follicular hyperkeratosis	7	7	2	0	2	8	0	5	2	4
Cheilosis	0	0.5	0	0	0	0	0	0	0	1
Glossitis	2	0.5	6	10	5	10	12	4	2	6
Abnormal tongue color	1	0.5	8	10	8	7	16	5	6	7
Edentulous*	0	0	38	10	27	19	30	17	31	23
Abnormal color and texture of hands	2	1	18	10	12	10	18	10	20	6
Corneal vascularity	0.5	0	0	0	2	1	1	0.5	1	2
Paresthesia	0	0	12	0	8	4	5	4	8	6

\*Not recorded as a sign of deficiency, but only to help interpret tongue changes.

the adults whose weight reduction below the U. S. standard was more marked. Thus, the averages for adults were below the U. S. standard by from 7 to 12 kilograms.

In addition to comparing averages for adults from 21 to 60 years of age, a distribution curve was constructed showing the frequency of occurrence of overweight and underweight. Each individual's weight was compared with the U. S. standard for age, sex, and height, and the number of kilograms below or above the standard was plotted on the frequency distribution curve (Fig. 1). The mean was -9 kilograms, and the mode, -11 kilograms. The reduction below the U. S. standard for weight becomes more striking when it is recognized that almost half (48 per cent) of those examined were more than 10 kilograms below the U. S. standard.

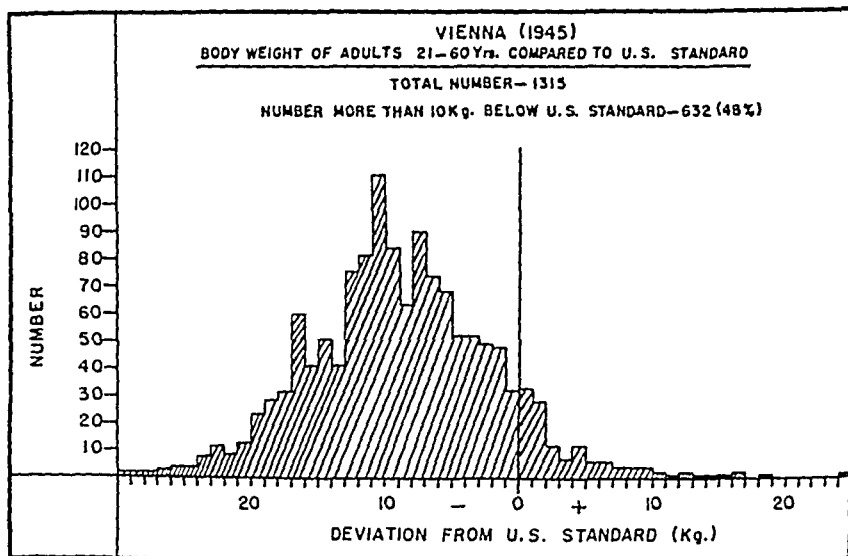


Fig. 1.

*Symptoms and General Appearance:* Although not specifically tabulated, many individuals spontaneously complained of weakness, fatigue, and even syncope. It was not unusual to see an ecchymosis or a "black eye" and, upon inquiring, it was frequently found that it had followed a fainting spell due to weakness.

The general appearance of the individuals was not that of extreme under-nutrition but simply of weight loss. Many appeared normal, a few, plump, but most showed evidence of considerable loss of weight and a few showed extreme weight loss. In the latter, posture was often poor, movements were carried out with a characteristic lethargy, and the facial expression was apathetic. Abnormal pigmentation was not seen.

*Symptoms and Signs Suggestive of Specific Deficiency States:* Severe vitamin deficiencies were not seen. There was no evidence of beri-beri, peripheral neuritis, or of pellagra, and only one individual with classic scurvy was seen. Certain signs that have been described as suggesting specific deficiencies were

recorded as they were seen. The low incidence of most of these signs is given in Table III. Several signs deserve comment.

Goitre, diffuse, nontoxic, was present in from 21 to 42 per cent of the individuals. It is not known whether this incidence is higher in Vienna at this time than before the war.

Distinct papillary atrophy of the tongue was seen in 156 instances. However, in sixty of these the mouth was edentulous so that the significance of the finding in these individuals is open to question. A beefy red or a magenta colored tongue was observed in 404 individuals of whom 102 were edentulous. The combination of papillary atrophy and either beefy red or magenta colored tongue was seen in thirty-two instances, seventeen being edentulous. Other signs suggesting thiamin, riboflavin, or niacin deficiency were almost absent.

*Paresthesia and Acrocyanosis:* In studying a small group of starved men we described a condition of the extremities, especially the hands, resembling acrocyanosis, which was frequently associated with paresthesia.<sup>8</sup> The same phenomenon, although with a lower incidence (from 10 to 20 per cent of the adults), was observed in the studies reported here. In the morning, particularly if cold, the hands and feet were cold to the touch and presented a mottled cyanotic appearance. In the warmer part of the day the cyanosis might give way to a mottled reddish flushing, but the hands were usually still cold to the touch. Paresthesia of the hands and feet was associated with acrocyanosis in only forty instances, so that the correlation of the two was not as striking as that found in starved prisoners. The paresthesia was described variously as numbness, tingling, or, often, a burning sensation. These symptoms were most marked in the finger tips and next in the toes; they even extended into the forearms and lower legs.

*Edema and Serum Proteins:* The serum protein concentration was determined in 859 individuals. The average values for the age and food ration categories are given in Table IV. The individual determinations are plotted as a distribution curve in Fig. 2. It is at once clear that the majority of the serum protein determinations were either normal or slightly reduced and hence the averages were only slightly below normal. Nevertheless, a significant number of individuals were found to have hypoproteinemia. In fact, 28 per cent of the values were 5.5 Gm. per 100 c.c. or below, and over fifty individuals had serum protein concentration between 4.5 and 5.0 Gm. per 100 cubic centimeters. The

TABLE IV. AVERAGE SERUM PROTEIN CONCENTRATION (GM. PER 100 C.C.)

FOOD RATION CLASSIFICATION	AGE	MALE		FEMALE	
		SERUM PROTEIN	NUMBER	SERUM PROTEIN	NUMBER
Heavy workers	19-40	6.8	9	6.3	3
	41-65	6.0	34	6.5	3
Workers	19-40	6.5	26	6.4	64
	41-65	6.4	89	6.5	53
Employees	19-40	6.3	9	6.3	37
	41-65	6.2	32	6.3	41
Others	19-40	6.6	14	6.5	97
	41-65	6.4	35	6.3	203
Children	1-18	6.3	37	6.5	36

hypoproteinemia, although not general, was present in an undoubtedly larger than normal proportion of the population studied.

Dependent edema was observed in 133 individuals. The data are given in Table V. In eighty-eight of these there was an obvious clinical cause for the edema, such as known or evident cardiac or renal disease, or varicose veins. There were forty-five individuals with edema in whom no clinical cause could be found and who were considered to have "hunger edema." The average age of these two groups was the same, but average weight loss was definitely more in the "hunger edema" group. Likewise the serum proteins, although not excessively low, were definitely lower in the latter group. There were also more signs suggestive of other specific deficiencies in the latter group.

*Anemia:* Blood hemoglobin concentration was determined in 859 individuals. The average values for the age and food ration categories are given in

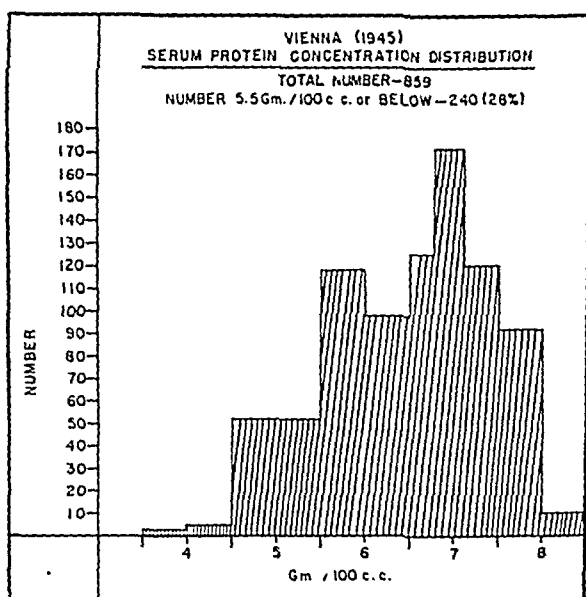


Fig. 2.

TABLE V

	EDEMA	
	WITH OBVIOUS CLINICAL CAUSE	WITHOUT OBVIOUS CLINICAL CAUSE
Number	88	45
Average age	54	54
Males	13	14
Females	75	31
Below U. S. standards (Kg.) (Av.)*	9.2	13.3
Average hemoglobin (Gm./100 ml.)	12.3	13.0
Average serum protein (Gm./100 ml.)	6.4	5.8
Clinical signst (%)	63	86
Total	133	

\*Compared with U. S. standards in War Department Training Manual 8-500.<sup>c</sup>

†Cheilosis, glossitis with atrophy, paresthesia, tongue red or violet in color.

Table VI. The individual determinations are plotted as a distribution curve in Fig. 3. Marked anemia was present in but a very small number. Nevertheless, the average values were definitely below normal. Only in the young male white collar workers was the value above 14 Gm. per 100 cubic centimeters. Thus it appears evident that a mild but definite anemia was present; indeed, a relatively small proportion of the individuals had hemoglobin concentrations of 15.5 Gm. per 100 c.c. or over.

TABLE VI. AVERAGE BLOOD HEMOGLOBIN CONCENTRATION (GM. PER 100 C.C.)

FOOD RATION CLASSIFICATION	AGE	MALE		FEMALE	
		HEMOGLOBIN	NUMBER	HEMOGLOBIN	NUMBER
Heavy workers	19-40	12.9	9	13.4	3
	41-65	13.7	34	13.2	3
Workers	19-40	13.7	26	13.2	64
	41-65	13.4	89	12.7	53
Employees	19-40	14.5	9	13.4	37
	41-65	13.1	32	12.5	41
Others	19-40	13.0	14	12.8	97
	41-65	13.3	35	13.2	203
Children	1-18	12.8	37	13.0	36

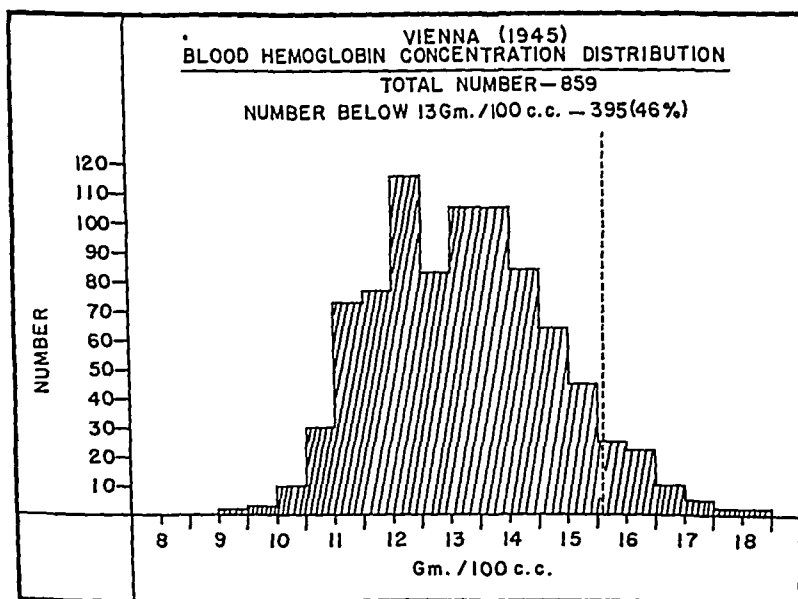


Fig. 3.

*Amenorrhea:* Absent menses of two months' duration or more, not due to pregnancy, was present in 16 per cent of the women between the ages of 15 and 44. The duration of absence was two to three months in 10 per cent, three to six months in 5 per cent, and more than six months in 1 per cent.

*Food Consumption.*—

The food consumption of 918 individuals was determined; that of 346 of those from bezirks 7, 8, and 9 is presented in Table VII.

TABLE VII. AVERAGE FOOD CONSUMPTION BY RATION GROUPS, VIENNA, BEZIRKS 7, 8, AND 9, AUGUST 27-31, 1945 (GRAMS PER PERSON PER DAY)

RATION GROUP	NUMBER	BREAD	FLOUR	FATS	POTATOES	GREEN VEG.	OTHER VEG.	LEGUMES	FRUIT	MILK	MARSHMALADE	SUGAR	BUTTER
Heavy workers and Workers													
Male	47	267	34	2	183	66	37	84	2		1	1	23
Female	12	332	65	3	104	50	17	58	63			1	
Employees													
Male	34	224	56	2	183	50	38	103	3		2	1	
Female	34	220	66	1	121	22	36	91	2		1	1	9
Children													
0 to 6	24	249	35	1	198	8		77		200			
6 to 12	31	337	32	1	177	12		44		16		8	
Others													
Male	17	221	48	3	215		44	69			1	2	
Female	147	206	39	2	124	24	57	77	6	6		1	6
Total	346												

Although meat, fats, and sugar were authorized for issue, there was no meat available and very little fat or sugar. Most families had none of these items. Likewise, there were no eggs available, with the exception of one individual in an outlying bezirk who reported having had one egg; there was almost no cheese. Consequently, the diet consisted principally of bread, or flour, potatoes, and pulses (dried beans or peas) with some fresh vegetables. Contrary to expectations, the vegetable consumption was not higher in the outlying bezirks (except in the case of the children) with the exception of a few cases where pregnant women or nursing mothers were able to obtain small amounts. The significant fact is that all of the protein of the diets of the people in bezirks 7, 8, and 9 were derived from vegetable sources, and this was also true, with a few exceptions, for the other groups surveyed.

It is also to be noted that ration supplements issued for the worker and heavy worker groups were obviously shared with the entire family, since the bread consumption for children was above the ration allowance, while that for the worker categories was below their ration allowance. This is quite evident when flour is considered a part of the bread ration.

The analyses of the food consumed by ration groups, as found in bezirks 7, 8, and 9, are presented in Table VIII.

It is apparent that the diets as consumed were quite deficient in calories, with the possible exception of children up to six years. Eighty-two per cent of the calories were provided from carbohydrates; whereas it is considered that good nutrition requires not more than 60 per cent of the calories provided from carbohydrates, with the balance from fat and protein. In these diets, fat is notably deficient.

The diets were also deficient in calcium, and in vitamin A riboflavin, and niacin. In most cases the vitamin C content is well below the recommended levels, but, with the exception of the diets for the children in bezirks 7, 8, and 9, the deficiency of vitamin C was probably not serious.

TABLE VIII. ANALYSIS OF FOOD CONSUMED BY RATION GROUPS; BEZIRKS 7, 8, AND 9, AUGUST 27-31, 1945

RATION GROUP	CALORIES	PROTEIN (GM.)	FAT (GM.)	CARBOHYDRATE (GM.)	CALCIUM (MG.)	IRON (MG.)	VIT. A ( $\frac{1}{2}$ CAROTENE VALUE)	VIT. B <sub>1</sub> (MG.)	VIT. B <sub>2</sub> (MG.)	NIACIN (MG.)	VIT. C (MG.)
Heavy workers and Workers											
Male	1190	50	6	242	240	15	2360	1.3	0.7	6.4	67
Female	1350	51	9	271	208	14	1850	1.3	0.6	6.1	47
Employees											
Male	1230	54	7	241	257	16	1900	1.0	0.7	6.7	59
Female	1150	50	16	228	180	15	410	1.3	0.6	5.8	38
Children											
0 to 6	1214	52	10	232	383	13	420	1.3	0.8	6.0	26
6 to 12	1260	44	6	253	144	12	400	1.3	0.8	6.0	24
Others											
Male	1100	44	7	219	162	13	430	1.2	0.6	5.4	39
Female	1000	43	6	200	202	12	1270	1.1	0.6	5.1	48

## DISCUSSION

From the data presented it is clear that the people of Vienna, as seen in the late summer of 1945, were grossly undernourished. This was evidenced by their appearance, weight reduction (48 per cent were more than 10 kilograms under the U. S. standard (Fig. 1), mild anemia, moderate hypoproteinemia, and "hunger edema" in a small but definite number of persons. It is also clear that the food being eaten at the time of the survey was not sufficient to maintain weight and adequate nutrition. Thus, one can from these facts suspect that frank starvation would have ensued at least by midwinter unless more food was provided. Furthermore, it is probable that with the low vitamin content of the diet some avitaminosis might also be expected. Shipments of food began to arrive, however, and it is reported that this continued throughout the winter and spring of 1946 in sufficient quantities to prevent starvation.

Certain of the observations deserve special comment. The low incidence of the signs associated with specific vitamin deficiencies is a remarkable observation and, in general, agrees with most other reports from Europe.<sup>1, 9</sup> Three factors may help account for this. First, the duration of the very poor diet may not have been long enough to permit the development of signs of deficiency; second, although low in vitamin content, the food did contain some of the B complex, although little C; and third, the metabolic rate was probably significantly reduced. This, as well as the low caloric intake, probably reduced the vitamin requirements.

Loss of weight of adults was by far the most striking finding in the city of Vienna. Nutrition surveys also were made in two smaller cities, Linz (population 250,000) and Enns (population 17,000), by the same group of investigators. In Linz, 22 per cent of the population was more than 10 kilograms in weight under the U. S. standard, in Enns, 18 per cent, as compared with 48 per cent in Vienna. Thus it may be seen that the large city suffered,

as one might expect, much more than the small ones. In a group of German prisoners,<sup>8</sup> 70 per cent were more than 10 kilograms below the U. S. standard. Here frank starvation was present and the incidence of hunger edema was 24 per cent.

It is a little surprising that the average weight of the children was not greatly below the U. S. standard. A similar survey made in 1920 and reported by the American Relief Administration<sup>9</sup> reported values for 10- and 15-year-old children that were lower than those given here, both for weight and height (Table II). It seems probable, therefore, that the nutritional status of the children during World War II was somewhat better than during World War I, and reflects the general increase in height and weight in children in many countries following the first war.

The high incidence of weakness, fatigue, and syncope is not surprising considering the extreme weight loss in many of the adults. The poor posture and lethargy have often been observed in severe weight loss.

It is difficult to interpret the occurrence of papillary atrophy and redness of the tongue since these signs occurred largely in edentulous individuals. Incidence of these signs does not necessarily signify a deficiency in vitamin B complex.

An acrocyanotic appearance of the hands, often with paresthesia, was definite but occurred in a much smaller proportion of the population than was observed in a group of starving German prisoners.<sup>8</sup> This suggests that the phenomenon is related to the degree of undernutrition. Others have described somewhat similar neurological manifestations in undernourished and starved groups and the data have been reviewed recently by Denny-Brown.<sup>10</sup>

The appearance of hunger edema, although in very small incidence, suggests that the nutrition of the general population was at a critical level. The average serum protein concentration was somewhat lower in this group than in those with other forms of edema or in those without edema.

The incidence and duration of amenorrhea in women between the ages of 15 and 44 is probably somewhat increased above that seen in normal individuals in normal times, and is probably related to a number of factors, including undernutrition and stress.

#### SUMMARY

A survey of the nutritional status of 2,325 Viennese civilians under United States occupation in the summer of 1945 revealed:

1. Marked reduction in weight, especially in adults, below the U. S. standard for height and weight.
2. Commonly observed weakness, easy fatigability, and even syncope; apathetic facial expressions.
3. Surprisingly few signs suggestive of specific vitamin deficiencies.
4. An acrocyanotic appearance of the hands and feet, often associated with paresthesia.
5. Hunger edema in forty-five individuals, although average serum protein concentrations were not far below normal.



6. Average hemoglobin concentrations below normal, although marked anemia was present in but a small number.

Analysis of the average food consumption at the time of the survey showed an intake of from 1,000 to 1,440 calories with about 50 Gm. of protein per day.

## REFERENCES

1. Davidson, C. S.: Nutrition and Food Supply in Germany and Austria, *Nutrition Rev.* 4: 161-163, 1946.
2. Pyke, M.: Nutrition in Vienna in September, 1945, *Brit. M. J.* 2: 839, 1945.
3. Salzer, M.: Mitteilungen der American Relief Administration; Vienna, August, 1921.
4. A Review of the Work of the American Relief Administration in Austria; Vienna, November, 1921.
5. Lowry, O. H., and Hastings, A. B.: Histochemical Change Associated With Aging. I. Methods and Calculations, *J. Biol. Chem.* 143: 257, 1942.
6. War Department Training Manual, 8-500, Washington, D. C., 1945, U. S. Government Printing Office.
7. Bridges, M. A., and Mattice, M. R.: Food and Beverage Analyses, Philadelphia, 1942, Lea & Febiger.
8. Davidson, C. S., Wilcke, H. L., and Reiner, P. J.: A Nutritional Survey of Starvation in a Group of Young Men, *J. LAB. & CLIN. MED.* 31: 721, 1946.
9. Stare, F. J.: Nutritional Conditions in Holland, *Nutrition Rev.* 3: 226, 1946.
10. Denny-Brown, D.: Neurological Conditions Resulting From Prolonged and Severe Dietary Restrictions, *Medicine* 26: 41, 1947.

# FAILURE OF THE ISOLATED PERFUSED MAMMALIAN HEART TO DESTROY RENIN AND ANGIOTONIN

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IT IS generally assumed that the polypeptide, angiotonin, is the active pressor substance responsible for arteriolar constriction in experimental hypertension and that it is formed by the interaction of the proteolytic enzyme renin on a substrate of plasma globulin. Since the point of pressor activity seems to be directly on the arteriolar muscle, the question of fixation of the pressor material by the effector tissue arises.<sup>1</sup> It is technically difficult to measure fixation by arteriolar muscle, but cardiac muscle is a readily accessible similar material. Consequently, renin and angiotonin were separately perfused through the coronary system of isolated rabbit hearts to determine whether there was any loss of either substance, and saline extracts of perfused and unperfused rabbit hearts were assayed for pressor activity.

Hog renin was prepared from hog kidney powder by the method of Schales.<sup>2</sup> The first or unpurified fraction of renin was used. This renin, which contained 95 mg. per cent total nitrogen, was relatively stable in the refrigerator and its activity was assayed at four cat units per cubic centimeter.\* This renin was diluted to 0.5 and 0.05 cat units per cubic centimeter with Ringer-Locke's solution. Rabbits were rendered unconscious by a blow on the head and the hearts were removed and washed in physiologic saline solution at a temperature of 37.6° C. The hearts were immediately attached to a modified Langendorff coronary perfusion apparatus and perfused with Ringer-Locke's solution until they were free from blood, at which time perfusion with renin or angiotonin was begun. Samples of the perfusate as it issued from the venous side were collected at approximately fifteen-minute intervals thereafter until forty-five minutes had elapsed.

The venous perfusate was assayed in cats anesthetized with pentobarbital sodium. Blood pressure changes were read directly from a mercury manometer attached by rubber tubing to a cannulated carotid artery and filled with 4 per cent sodium citrate. The solutions to be assayed were injected into a femoral vein. The higher concentrations of renin were assayed directly as collected from the coronary system, but the more dilute solutions were first converted to angiotonin by the method of Schales, Holden, and Schales.<sup>4</sup> The angiotonin perfusate was also prepared by this method, beef plasma being used as a source of globulin substrate, and was so diluted with Ringer-Locke's solution that 1.0 c.c. gave a 20 mm. rise of blood pressure in the assay cats.

From the Research Laboratories of the Alton Ochsner Medical Foundation.

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Renin and angiotonin were kindly prepared for us by Otto Schales, D.Sc.

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\*One cat unit equals the amount of renin which, if injected intravenously in the anesthetized cat, will produce a blood pressure rise of 30 mm. of mercury.

Different rabbit hearts were used for each dilution of renin and angiotonin. All the perfused hearts were frozen in the refrigerator for varying periods. After refrigeration the hearts were cut into small pieces and ground in a mortar. Each minced heart was then shaken with 20 c.c. of physiologic saline solution for thirty minutes, the mixture centrifuged, and the supernatant fluid assayed for pressor activity in the described manner. Unperfused rabbit hearts were treated and assayed in the same fashion.

Equivalent pressor responses were obtained by the concentrated renin solution before and after perfusion through the isolated rabbit heart (Table I, Experiments 2, 3, 4, and 6). Similarly, following conversion of the dilute renin solution to angiotonin no difference in pressor activity could be demonstrated after perfusion (Table I, Experiments 1 and 5). Equivalent pressor responses were also obtained with angiotonin both before and after perfusion (Table II).

TABLE I. ASSAY OF RENIN IN CORONARY PERFUSATE (INJECTIONS MADE IN ANESTHETIZED CAT)

EXPERIMENT	INJECTION TEST MATERIAL	INJECT. (C.G.)	B. P. RISE (MM. HG)	RENIN LOSS
1	Ringer-Locke's solution	1	0	None
		1	0	
	Unperfused renin, 0.05 cat unit/c.c., converted to angiotonin	2	25	
		1	15	
	Perfused renin, 0.05 cat unit/c.c., converted to angiotonin	2	25	
		2	20	
		2	25	
2	Ringer-Locke's solution	2	0	None
		2	30	
	Unperfused renin, 0.5 cat unit/c.c.	2	30	
	Perfused renin, 0.5 cat unit/c.c. No. 1	2	30	
		2	20	
	No. 2	2	30	
		2	20	
3	Ringer-Locke's solution	2	0	None
		2	0	
	Unperfused renin, 0.5 cat unit/c.c.	2	25-30	
	Perfused renin, 0.5 cat unit/c.c.	2	30	
		2	30	
4	Ringer-Locke's solution	1	0	None
		1	0	
	Unperfused renin, 0.5 cat unit/c.c.	2	30	
		2	30	
	Perfused renin, 0.5 cat unit/c.c. No. 1	2	30	
	No. 2	2	30	
		2	30	
	No. 3	2	25-	
5	Inactivated angiotonin	2	0	None
		2	0	
	Unperfused renin, 0.05 cat unit/c.c., converted to angiotonin	2	18	
		2	12	
	Perfused renin, 0.05 cat unit/c.c., converted to angiotonin	1	10	
		2	20	
		2	15+	
6	Ringer-Locke's solution	2	0	None
		2	0	
	Unperfused renin, 0.5 cat unit/c.c.	2	30	
	Perfused renin, 0.5 cat unit/c.c.	2	25-30	
		2	30	

TABLE II. ASSAY ON ANGIOTONIN IN CORONARY PERFUSATE (INJECTIONS MADE IN ANESTHETIZED CAT)

EXPERIMENT	TEST MATERIAL	INJECT. (C.C.)	B. P. RISE (MM. HG.)	ANGIOTONIN LOSS
7	Ringer-Locke's solution	2 2	0 0	None
	Unperfused angiotonin 1:20 in Ringer-Locke's solution	1 1	20 20	
	Perfused angiotonin 1:20 in Ringer-Locke's solution			
	No. 1	1 1	20 15-20	
	No. 2	1 1	20 20	
	No. 3	1 1	20 20	
8	Ringer-Locke's solution	1	0	None
	Unperfused angiotonin 1:20 in Ringer-Locke's solution	1 1	20 20	
	Perfused angiotonin 1:20 in Ringer's Locke's solution			
	No. 1	1 1	15-20 20	
	No. 2	1 1	20 20	
	No. 3	1 1	20 20	

Saline extracts of both the perfused and the unperfused rabbit hearts were then injected into assay cats and they produced identical pressor responses. Each kind of extract caused an initial drop in blood pressure of 10 to 15 mm. of mercury followed by a rapid rise of 10 to 15 mm. above its original level which lasted less than two minutes. It is assumed that this pressor response is due to the epinephrine-like compound described by von Euler<sup>7</sup> and others.

Renin in a concentration of 0.5 cat unit per cubic centimeter, when perfused through the rabbit heart, caused a decrease in the rate and amplitude of ventricular contraction. On the other hand, a solution whose concentration was 0.05 cat unit per cubic centimeter had no demonstrable effect on the heart. It is believed that the toxic effect of the more concentrated renin was due to the toluene preservative or to other foreign material and not to the renin itself, since others<sup>8</sup> have shown that renin is without effect on the mammalian heart and that toluene in Ringer-Locke's solution is toxic.

Angiotonin, in the concentration used, transiently diminished coronary flow, but a slight augmentation in the rate and amplitude of ventricular contraction was noted. These observations corroborate the work of others.<sup>5, 6</sup>

#### CONCLUSIONS

1. Renin and angiotonin may be perfused through the coronary system of the rabbit without loss of pressor activity.

2. Saline extracts of normal rabbit hearts and of rabbit hearts which have been perfused with either renin or angiotonin exhibit slight but equal pressor responses.

## REFERENCES

1. Landis, E. M., Thomas, E. D., and Wood, J. E., III: Comparative Pressor Activity of Blood Plasma Removed During Slow Infusions of Epinephrine and Renin, *Federation Proc.* 4: 43, 1945.
2. Schales, O.: Preparation and Properties of Renin, *J. Am. Chem. Soc.* 64: 561, 1942.
3. Smith, R.: Personal communication.
4. Schales, O., Holden, M., and Schales, S. S.: Renin-Substrate From Beef Serum, *Arch. Biochem.* 2: 67, 1943.
5. Hill, W. H. P., and Andrus, C. E.: Effects of Renin and of Angiotonin Upon Isolated Perfused Heart, *Proc. Soc. Exper. Biol. & Med.* 44: 213, 1940.
6. Lorber, V.: The Action of Angiotonin on the Completely Isolated Mammalian Heart, *Am. Heart J.* 23: 37, 1942.
7. von Euler, U. S.: The Presence of a Sympathomimetic Substance in Extracts of Mammalian Heart, *J. Physiol.* 105: 38, 1946.

# POTASSIUM CONTENT OF NORMAL CEREBROSPINAL FLUID

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WITH THE TECHNICAL ASSISTANCE OF M. LUCILLE KEEFER

SINCE the investigations of Mestrezat,<sup>1, 2</sup> reported in 1911 and 1912, the concentration of most of the inorganic elements in normal cerebrospinal fluid has been well known. During the ensuing three decades many workers improved the methods of analysis, adapted them for clinical application, and confirmed the reported concentrations of inorganic elements. The concentrations of the cations sodium, calcium, magnesium, and phosphorus have been well established. The levels of the anions chloride, phosphate, and bicarbonate also can be stated without qualification. The level of the cation potassium, however, has remained more or less in question. For many years the determination of this ion has been of academic interest only, and possibly is even now. For its determination various workers have employed methods derived from chemical reactions which are not ideal for quantitative analysis. Values have been reported after the study of small groups of incompletely described patients. Merritt and Smith<sup>3</sup> concluded that, "the potassium content of the cerebrospinal fluid has not been sufficiently investigated."

In Table I the results obtained by workers who have examined normal spinal fluids have been tabulated. In most cases there was no statement of the estimated accuracy of the method employed, nor has it been possible in every case to ascertain the method used. It may be questioned whether the number of determinations made constituted an adequate series for the formation of statistically significant conclusions.

TABLE I. POTASSIUM CONTENT FOUND BY OTHER INVESTIGATORS

INVESTIGATOR	NUMBER OF CASES	RANGE (MG. PER 100 ML.)	AVERAGE (MG. PER 100 ML.)	METHOD OF ANALYSIS
Leulier, Velluz, and Griffon <sup>4</sup>	20	11 -16	12.3	Modified cobaltinitrite
Pincus and Kramer <sup>5</sup>	3	12.8-16.6	15.1	Cobaltinitrite
Leipold <sup>6</sup>	16	10.5-16.9	13.5	Not stated
Merritt and Smith <sup>3</sup>	15	11 -15	13.2	Not stated
Honeyman and Zwemer <sup>7</sup>	10	12 -14.5	13.3	Modified cobaltinitrite
Eisler <sup>8</sup>	19	13 -13.5	13.2	Cobaltinitrite
Lierle and Sage <sup>9</sup>	4	not stated	12.8	Cobaltinitrite
Duliere <sup>10</sup>	14	not stated	11.17	Cobaltinitrite
Ballif and Gherseovici <sup>11</sup>	not stated	10 -18	not stated	Cobaltinitrite

## MATERIAL

The samples of spinal fluid were taken from the lumbar subarachnoid space of patients entirely free from clinical evidence of neurologic disorders. (McCance and Watchorn,<sup>12</sup> in a small series, found that the potassium content of ventricular fluids was slightly less than that

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of fluid from the lumbar sac. Unpublished data of our own indicate a difference in the content at the central and at more distal levels in the same patients.) The ages varied widely from the first to the fifth decade. The number of male and female patients was approximately equal. Many of the specimens were obtained at the time of administration of a spinal anesthetic; that is, the fluid was removed before the agent was instilled. The occasion for the anesthesia was an elective operation such as a hernioplasty. Most of these patients had been fasting twelve hours, but there was no apparent disturbance in fluid or electrolyte balance. The remaining specimens were obtained from patients convalescing on surgical wards. Care was taken to use only those who were in satisfactory general health and without fluid or electrolyte imbalance. No fluid was used from any patient who had a positive blood Kahn reaction.

The fluid was collected in chemically clean test tubes with aseptic precautions. It was placed in the refrigerator until the analysis could be begun. Microscopic examination of the fluid was not made in every case. Ballif and Ghercoviei<sup>11</sup> state that the mixture in vitro of cerebrospinal fluid with the erythrocytes of the same individual did not affect the potassium content of the cerebrospinal fluid, even when the mixture was kept from one to four days. All fluids examined in this study were crystal clear.

As a precaution, all fluids were ashed within forty-eight hours of their removal from the patient. It was believed that delay after this treatment of the sample would not be accompanied by any quantitative alteration of potassium content.

It is beyond the scope of this work to criticize the various methods available for the determination of potassium in biological fluids. Peters and Van Slyke, in addition to other authorities, have indicated that the determination of potassium by converting it to potassium chloroplatinate and subsequently iodoplatinate is a method based on sound principles of quantitative analysis. The Lindo-Gladding procedure<sup>13</sup> was adapted by Shohl and Bennett<sup>14</sup> and later altered by Consolazio and Talbott.<sup>15</sup> We have employed their methods with few alterations. The changes made, however, seemed of such importance that we have detailed our steps. We have borrowed freely from Shohl and Bennett and from Consolazio and Talbott for the general outline of the analysis.

#### METHOD

The principle of the method is the precipitation of potassium chloroplatinate in the ash of biologic fluids and its determination by titration. A quantitative determination is made possible by the conversion of potassium chloroplatinate to potassium iodoplatinate on the addition of potassium iodide. The wine color of potassium iodoplatinate varies in intensity depending upon the amount of potassium chloroplatinate reacting with potassium iodide. The rate of development of the color is proportional to the concentration of the potassium iodide. Heat also speeds the development of color. In the presence of acid color development is hastened, but an excess of acid liberates free iodine which will destroy the proportional relation of color development. Sunshine will also liberate iodine.

In the final titration iodoplatinate is reduced in neutral solution with sodium thiosulfate. This reduced salt is lemon colored and serves as a self-indicator. At the end point one drop of the thiosulfate changes the solution's color from reddish- to greenish-yellow.

#### *Reagents and Special Equipment.*—

50 per cent sulfuric acid.

50 per cent sodium sulfate solution.

20 per cent chloroplatinic acid in N HCl. Made by dissolving 10 Gm. platinum chloride,  $\text{H}_2\text{PtCl}_6 \cdot 6\text{H}_2\text{O}$  in N hydrochloric acid and diluting to 50 ml. with N HCl.

Absolute ethyl alcohol saturated with potassium chloroplatinate. The potassium chloroplatinate was prepared by mixing 0.286 Gm. of potassium chloride, 0.5 Gm. of platinum chloride,  $\text{H}_2\text{PtCl}_6 \cdot 6\text{H}_2\text{O}$ , and 1 ml. distilled water. These were allowed to stand a short time; the supernatant was then drained off by suction, and the precipitate washed twice with 5 ml. of absolute ethyl alcohol. The precipitate was dried by evaporation and kept in a desiccator. To 500 ml.

absolute alcohol was added the amount of potassium chloroplatinate held on a toothpick end. This was shaken and allowed to stand overnight. Without disturbing the precipitate, the solution was filtered immediately before each use. The unused solution was returned to the bottle containing the excess of potassium chloroplatinate.

2 N (approximately) potassium iodide. This was made each time immediately before use and placed in a brown bottle.

0.1 N sodium thiosulfate. The normality required frequent checking.

0.1 N potassium iodate. This was used to standardize the sodium thiosulfate solution.

10 ml. capacity cone-pointed quartz tubes.

Glass stirring rods, 1 mm. diameter and 200 mm. long.

Muffle furnace.

Microburette with fine capillary tips for delivery.

Capillary suction tips for removal of supernatant without disturbing the precipitate.

### PROCEDURE

Each determination was completed in one cone-pointed quartz tube. Two blanks were prepared, each containing one drop 50 per cent sulfuric acid and one drop 50 per cent sodium sulfate. Wherever possible, samples were analyzed in duplicate or triplicate. One milliliter of unknown solution, one drop 50 per cent sulfuric acid, and one drop 50 per cent sodium sulfate were placed in the quartz tube. The tubes were kept in the constant temperature oven at 100 to 105° C. until the solution had evaporated; this process usually required four hours. The tubes were transferred to the sand bath with temperature at 100° C. Placing the tubes in the sand at an angle seemed to result in more even distribution of the heat. The sand bath temperature was slowly raised to 260° C. and maintained for not less than twelve hours. The tubes were transferred to a cold muffle furnace and the temperature slowly raised to 475 to 500° C. This temperature was maintained for four hours and it thoroughly ashed the tube contents, producing a fine white powder. During the process of ashing, the residue tended to creep up the sides of the quartz tubes. Slowing the ashing step controlled this creeping tendency and eliminated the error which would have resulted. The tubes were left in the furnace until cool. Results were better if the determination was continued from this stage without delay.

To each tube was added 0.2 ml. of 20 per cent chloroplatinic acid which dissolved all the ash. Five milliliters of filtered absolute alcohol saturated with potassium chloroplatinate were added; the solution was thoroughly mixed and allowed to stand for thirty minutes. The tubes were centrifuged for five minutes at 2,000 revolutions per minute. The supernatant liquid was removed through a capillary tube by suction. The procedure was carried out with gentle suction because a minute portion of the precipitate floats and does not settle during centrifuging. By keeping the capillary tip well submerged and moving it toward the precipitate in the quartz tube base as siphoning proceeds, all the supernatant fluid can be removed without loss of precipitate.

Five milliliters more of the filtered absolute alcohol were added to each tube and mixed well using the capillary stirring rod which accompanied the tube throughout the determination. All yellow color was washed from the precipitate. Each stirring rod in each quartz tube was rinsed with approximately 0.5 ml. of the alcohol. The tubes were again centrifuged and the supernatant removed by cautious suction. If any yellow color remained in the precipitate, the entire washing process was repeated. Only by washing out all the yellow chloroplatinic acid could an accurate final yield of potassium be obtained.

The tubes were placed in a water bath at 70° C. and slowly brought to boil; boiling was continued until all traces of alcohol were evaporated. After each stirring rod had been returned to its proper tube, the precipitate in each was dissolved in 5 ml. distilled water. To each tube was then added 1 ml. 2 N potassium iodide. The tubes were held in the hot bath a few minutes for full color development and then were placed in an ice bath and kept cold until titration was completed. It was found that the reduced tem-



perature limited the oxidation of the potassium iodoplatinate by atmospheric oxygen, rendering the potassium yield more accurate.

In the absence of direct sunlight, each tube was titrated to a lemon yellow with freshly made 0.01 N sodium thiosulfate. Just before use this was made from the 0.10 N standard sodium thiosulfate reagent. It was found that for accurate results it was necessary to complete the titration within thirty minutes after the addition of the potassium iodide solution.

It is of importance to know the accuracy obtainable by this analysis. Shohl and Bennett,<sup>14</sup> employing the chemical reactions outlined, found that 0.1 mg. could be determined  $\pm 4$  per cent, and 0.4 mg.,  $\pm 2$  per cent. Consolazio and Talbott,<sup>15</sup> modifying Shohl and Bennett's work so that the determinations were carried out in one container (a cone-pointed quartz tube) without transfer, believed their average error to be less than 2 per cent. By statistical analysis of the values obtained from determinations on replicate samples it was shown that in the method employed in this investigation the standard deviation was  $\pm 0.27$  mg. per cent. Expressed in per cent the analytical error of the method was 2.75.

### RESULTS

The results of the study are summarized in Table II. In analysis of crystal clear samples from sixty-four patients who were without fluid or electrolyte imbalance, detectable signs of organic central nervous system disease, or syphilis, the values were found to range from 8.5 to 11.5 mg. per cent. It is seen in Table I that other workers have found ranges differing somewhat from this. There are two differences which preclude comparison of their results and the results we obtained. The number of analyses completed are not of the same order. In most studies the cobaltinitrite method was employed and it is now accepted that in potassium sodium cobaltinitrite the potassium content may vary.

TABLE II. POTASSIUM CONTENT IN SAMPLES OF SPINAL FLUID FROM SIXTY-FOUR NORMAL HUMAN BEINGS (MG. PER 100 ML.)

8.5	9.3	9.8	10.3
8.7	9.3	9.8	10.3
8.8	9.3	9.8	10.3
8.9	9.4	9.9	10.4
8.9	9.4	9.9	10.4
9.0	9.4	10.0	10.5
9.0	9.5	10.0	10.5
9.0	9.5	10.0	10.5
9.1	9.6	10.0	10.6
9.1	9.6	10.1	10.6
9.1	9.6	10.1	10.7
9.1	9.6	10.1	10.7
9.1	9.6	10.1	10.7
9.2	9.7	10.2	10.9
9.2	9.7	10.2	10.9
9.3	9.8	10.2	11.5

Analysis of the normal values gave a mean of 9.8 mg. per cent. The standard deviation of this value was 1.1 mg. per cent; or expressing the standard deviation of the mean in per cent it was 11.2.

### SUMMARY

Brief reference to earlier investigations of the concentration of inorganic constituents of normal spinal fluid has been made. Samples of spinal fluid obtained by lumbar puncture from sixty-four normal people were examined for

potassium content. The steps of the analytical method were detailed; these included the formation of potassium chloroplatinate from the potassium contained in the unknown, the conversion of this salt to potassium iodoplatinate, and its final titration with sodium thiosulfate.

The results obtained from the sixty-four normal subjects were tabulated. The range of potassium was found to be 8.5 to 11.5 per cent. A mean of 9.8 mg. per cent was calculated.

Without the assistance of Dr. Henry Ryder it would have been impossible to complete the statistical analysis of results obtained in the preliminary evaluation of our method and its application to normal spinal fluid. Dr. Charles D. Stevens and Dr. Samuel Rappaport rendered invaluable assistance with many problems which arose as the method of chemical analysis was being perfected.

#### REFERENCES

1. Mestrezat, W.: *Etude du Liquide Céphalo Rachidien Normal; Composition Chimique et Nature Vraie*, Montpel. méd. 32: 610, 1911.
2. Mestrezat, W.: *Le Liquide Céphalo Rachidien Normal et Pathologique. Valeur de l'examen Chimique. Syndromes Humoraux Dans les Diverses Affections*, Paris, 1912, A. Maloine.
3. Merritt, H. H., and Smith, F.: *The Cerebrospinal Fluid*, W. B. Saunders Company, Philadelphia, 1937, p. 28.
4. Leulier, A., Velluz, L., and Griffon, H.: *Sur le teneur en potassium du liquide céphalo rachidien normal*, *Compt. rend. Soc. de Biol.* 99: 1748, 1928.
5. Pineus, J. B., and Kramer, B.: *Comparative Study of the Concentration of the Various Anions and Cations in the Cerebrospinal Fluid and Serum*, *J. Biol. Chem.* 57: 463, 1923.
6. Leopold, W.: *Durchlassigkeitsverhältnisse der Bluthirnschranke*, E. Panzig Co., Greifswald, 1928, p. 29.
7. Honeyman, W. M., and Zwemer, R. L.: *Cerebrospinal Fluid Potassium in Neurological Disorders*, *Bull. Neurol. Inst., New York* 3: 297, 1938.
8. Eisler, B.: *Über das Verhältnis von Kalium zu Calcium und den Magnesiumgehalt des Liquor Cerebrospinalis*, *Ztschr. f. d. ges. exper. Med.* 61: 549, 1928.
9. Lierle, D. M., and Sage, R. A.: *A Study of the Calcium Phosphorus and Potassium of the Serum and Spinal Fluid in Asthma*, *J. Allergy* 3: 325, 1931-32.
10. Duheré, W. L.: *Données Expérimentales sur la Répartition du Potassium Dans le Sang Extravasé; Comparaison Entre la Concentration Physiologique Dans le Plasma et la Concentration Dans le Liquide Céphalo-rachidien*, *Compt. rend. Soc. de Biol.* 108: 416, 1931.
11. Ballit, L., and Gherseovici, I.: *Le pH du Liquide Céphalo-rachidien et Dosage du Potassium Rachidien Dans le Diagnostic des Hémorragies Mningo-Encéphaliques*, *Compt. rend. Soc. de Biol.* 109: 232, 1932.
12. McCance, R. A., and Watchorn, E.: *Inorganic Constituents of Cerebrospinal Fluids—Ventricular and Loculated Fluids*, *Brain* 57: 333, 1934.
13. *Official and Tentative Methods of Analysis*, ed. 4, Washington, 1935, Association of Official Agricultural Chemists, p. 12.
14. Shohl, A. T., and Bennett, H. B.: *A Micro Method for the Determination of Potassium as Iodoplatinate*, *J. Biol. Chem.* 79: 55, 1927.
15. Consolazio, W. V., and Talbott, J. H.: *Modification of the Method of Shohl and Bennett for the Determination of Potassium in Serum and Urine*, *J. Biol. Chem.* 126: 55, 1938.

## EFFECTS OF TETRAETHYL AMMONIUM CHLORIDE ON A MIXED TYPE OF HYPERSENSITIVE CAROTID SINUS SYNDROME

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IT IS now well known that hypersensitive carotid sinus reflexes can cause syncope or convulsive seizures in human beings. Weiss and Baker<sup>1</sup> described three types of cardiovascular response to stimulation of a hypersensitive carotid sinus, all of which may cause loss of consciousness. The first of these occurs without changes in arterial blood pressure or pulse rate. It is accompanied by blanching of the face, which is believed to reflect cerebral vasoconstriction sufficient to cause temporary cerebral hypoxia. The second type, occurring particularly in persons with arteriosclerosis, is accompanied by syncope which is preceded by a marked fall in blood pressure with little or no change in pulse rate. The type of cerebral ischemia thus induced is due to arterial hypotension. These two types of response are thought to result from increased activity of the sympathetic nervous system. The third type, the most common, results from reflex increase of vagal tone. During this form of reaction there is marked slowing of the pulse rate without primary change in arterial pressure. The bradycardia and the accompanying cerebral manifestations can be prevented by adequate dosage of atropine sulfate.

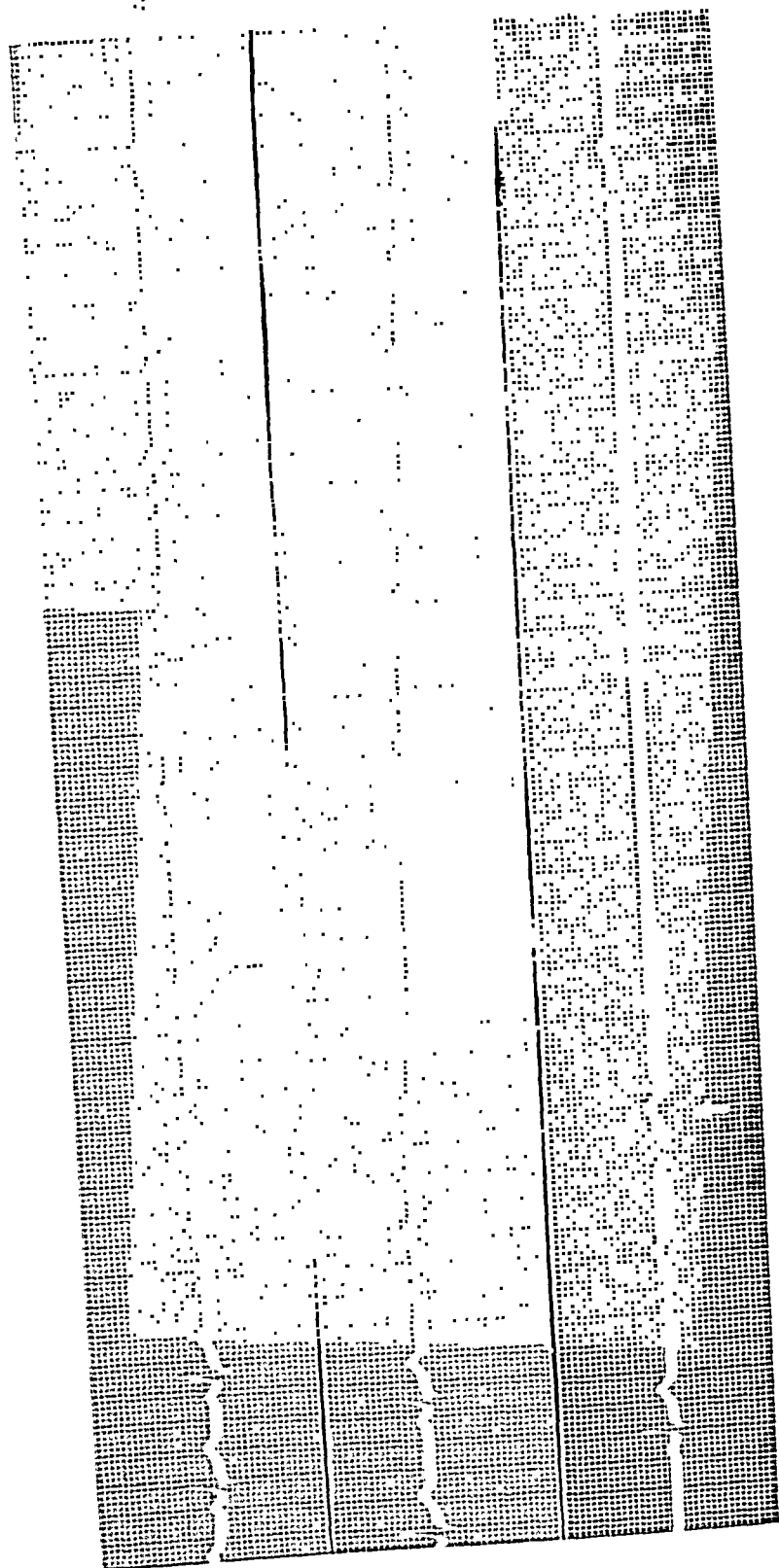
Recently, a patient who exhibited signs and symptoms of increased responsiveness to stimulation of the carotid sinus was referred to the Research Division of Cleveland Clinic Foundation. Because of the present interest in the effects of tetraethyl ammonium ion upon functions of the vegetative nervous system, the effect of the drug was investigated in this syndrome.

### CASE REPORT

A 52-year-old white man had noted over a period of months brief episodes of giddiness which followed stooping, sudden turning of the head, and shaving his neck. On one occasion he lost consciousness and had a short convulsive seizure. Examination showed grade 1 retinal arteriolar sclerosis. Arterial blood pressure was 130/80 at the first visit but thereafter averaged 110/70. Pressure applied to the left carotid sinus while the patient was sitting caused bradycardia and reproduced the giddiness. Pressure on the right sinus caused marked slowing of the heart and a brief, generalized, clonic seizure which ended when pressure was removed. Examination showed the blood and urine to be normal. The standard limb leads of the electrocardiogram were normal.

*Effects of Carotid Sinus Stimulation on Pulse Rate and Blood Pressure.*—Striking bradycardia resulted from pressure applied to either the right or left carotid sinus or to both. In one instance, asystole which was prolonged for 5.4 seconds was recorded in standard limb Lead II of the electrocardiogram (Fig. 1). The blood pressure during stimulation fell from control levels of 110/70 to range from 90/60 to 60/50. The degree of hypotension was dependent on the intensity and duration of pressure and on whether one or both sinuses were stimulated. It was thus regularly possible to reproduce the presenting symptom of giddiness.

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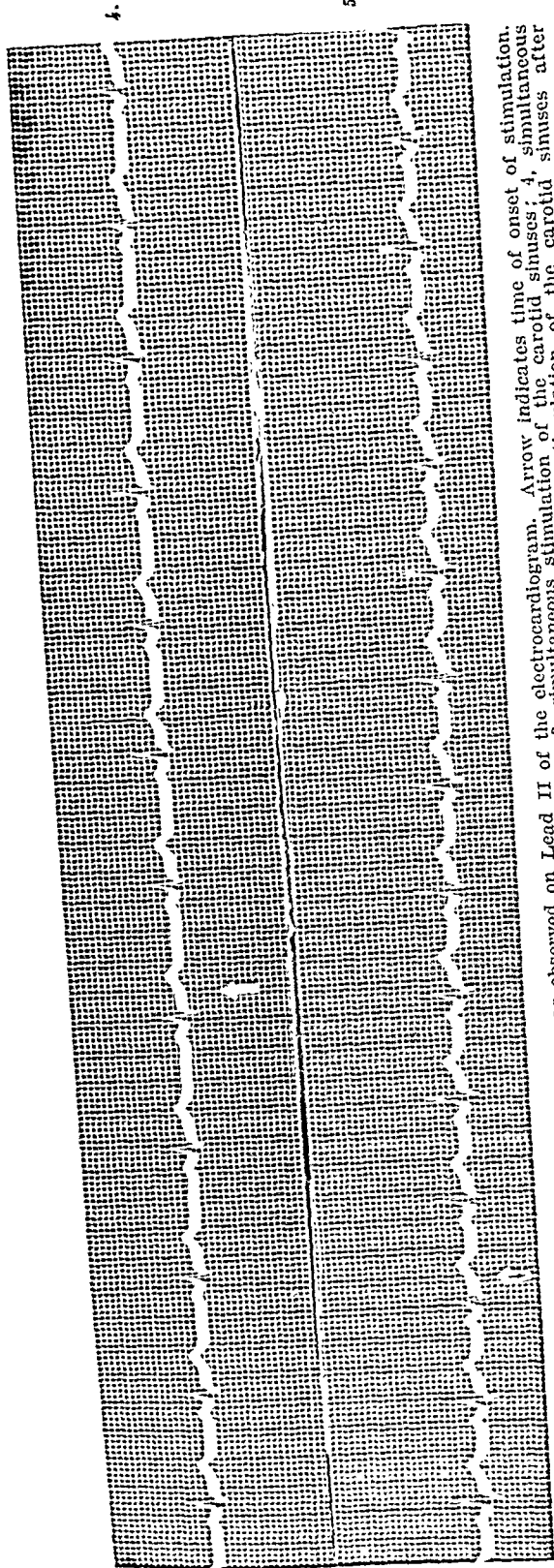


FIG. 1.—Effects of stimulation of carotid sinuses as observed on Lead II of the electrocardiogram. Arrow indicates time of onset of stimulation. 1, stimulation of the left carotid sinus; 2, stimulation of the right carotid sinus; 3, simultaneous stimulation of the carotid sinuses after stimulation of the carotid sinuses after intravenous injection of 1.3 mg. atropine sulfate; 5, simultaneous stimulation of the carotid sinuses after intravenous injection of 400 mg. tetrathyl ammonium chloride.

*Effects of Atropine Sulfate.*—After the intravenous injection of 1.3 mg. of atropine sulfate even severe stimulation of the sinuses did not slow the heart rate appreciably (Fig. 1). Although the blood pressure fell from 102/72 to 84/52, no subjective symptoms appeared (Table I). The persistence of a hypotensive response after atropine had prevented bradycardia indicates that the type of hypersensitivity was mixed, that is, of both second and third types of reaction.

TABLE I. EFFECTS OF STIMULATION OF CAROTID SINUSES BEFORE AND AFTER INTRAVENOUS INJECTION OF ATROPINE SULFATE AND OF TETRAETHYL AMMONIUM CHLORIDE

	CONTROL		AFTER INTRAVENOUS INJECTION OF 1.3 MG. ATROPINE SULFATE		AFTER INTRAVENOUS INJECTION OF 400 MG. TETRAETHYL AMMONIUM CHLORIDE	
	BLOOD PRESSURE (MM. HG.)	PULSE (PER MIN.)	BLOOD PRESSURE (MM. HG.)	PULSE (PER MIN.)	BLOOD PRESSURE (MM. HG.)	PULSE (PER MIN.)
Initial	104/70	83	102/70	84	90/60	91
During stimulation of left carotid sinus	98/66	55	88/68	82	90/60	91
During stimulation of right carotid sinus	68/48	(3.2 sec. asystole)	84/54	80	88/50	88
During simultaneous stimulation of both carotid sinuses	60/50	(5.4 sec. asystole)	84/52	70	88/50	85

*Effects of Tetraethyl Ammonium Chloride.*—Intravenous injection of 400 mg. of tetraethyl ammonium chloride reduced the arterial pressure on an average from 110/70 to 80/50-90/60 on three occasions. There was slight increase in pulse rate (82 to 90) and the only subjective symptoms were slight and transient numbness and tingling. Two to three minutes after administration of the drug, pressure over the carotid bifurcations caused no change in pulse rate, elicited no symptoms, and the blood pressure fell only 5 to 10 mm. systolic and diastolic. The refractory phase persisted for thirty to forty minutes, at the end of which time stimulation induced the phenomena seen in control observations.

Tetraethyl ammonium chloride (400 mg.) was given by intramuscular injection on alternate days for fourteen days. During this time, the symptoms did not recur. However, it was not unusual for several weeks to pass without symptoms when no treatment was being given.

#### COMMENT

The mechanism by which tetraethyl ammonium ion blocks the passage of impulse over the autonomic nervous system has been studied by Acheson and Moe.<sup>2, 3</sup> They demonstrated that the drug paralyzes sympathetic and parasympathetic ganglia. Clinical evidences of parasympathetic inhibition were furnished by Lyons and co-workers<sup>4</sup> who demonstrated gastrointestinal hypomotility and loss of tone of the urinary bladder. Birchall, Taylor, Lowenstein, and Page<sup>5</sup> attributed the mydriasis and dry mouth which followed intravenous injection of the drug to paralysis of the parasympathetic ganglia of cranial nerves.

In the light of this evidence it is not surprising that tetraethyl ammonium inhibits the increased vagal responsiveness of hypersensitivity of the carotid sinuses. In contrast to atropine, which acts at nerve endings, tetraethyl ammonium blocks transmission at the ganglia. Nevertheless, the ultimate effects of the two drugs on the parasympathetic component of the hypersensitive carotid sinus reflex are similar. This similarity of action does not extend to the sym-

pathetic aspect of the carotid sinus reflex, namely the hypotension. Atropine does not prevent the fall in blood pressure induced by sinus stimulation. Tetraethyl ammonium, by virtue of its blocking both parasympathetic and sympathetic ganglia, prevents both the vagal component of bradycardia and the arterial hypotension.

These observations furnish further clinical evidence of the activity of tetraethyl ammonium on the autonomic nervous system. However, the effects in hypersensitivity of the carotid sinus are transient. Consequently, the drug is not recommended as a substitute for atropine in the management of hypersensitivity of the carotid sinus, characterized by bradycardia.

#### SUMMARY

A 52-year-old man who had hypersensitive carotid sinus syndrome was observed. Stimulation of the sinuses by pressure caused bradycardia, reduction of blood pressure, giddiness and syncope. The bradycardia was presumably due to increased vagal tone and the fall of arterial pressure to vasodilatation of sympathetic origin. Atropine sulfate, by its ability to inhibit parasympathetic activity, blocked the vagal but had no effect upon the sympathetic component. Tetraethyl ammonium chloride, which paralyzes both parasympathetic and sympathetic ganglia, prevented both responses. This inhibition of the vegetative nervous system by tetraethyl ammonium persisted for only thirty to forty minutes. Because the effect of tetraethyl ammonium, though more complete than that of atropine, is of brief duration, it is not recommended for treatment of hypersensitive carotid sinus syndrome of this type.

We are indebted to Dr. E. C. Vonder Heide, Parke, Davis & Company, Detroit, Mich., for a generous supply of tetraethyl ammonium chloride (Etamon).

#### REFERENCES

1. Weiss, S., and Baker, J. P.: The Carotid Sinus Reflex in Health and Disease: Its Role in Causation of Fainting and Convulsions, *Medicine* 12: 297-354, 1933.
2. Acheson, G. H., and Moe, G. K.: Some Effects of Tetraethyl Ammonium on Mammalian Heart, *J. Pharmacol. & Exper. Therap.* 84: 189-195, 1945.
3. Acheson, G. H., and Moe, G. K.: The Action of Tetraethyl Ammonium Ion on the Mammalian Circulation, *J. Pharmacol. & Exper. Therap.* 87: 220-236, 1946.
4. Lyons, R. H., Moe, G. K., Neligh, R. B., Hoobler, S. W., Campbell, K. N., Berry, R. L., and Kennick, B. R.: The Effects of Blockade of the Autonomic Ganglia in Man With Tetraethyl Ammonium. Preliminary Observations on Its Clinical Application, *Am. J. M. Sc.* 213: 315-323, 1947.
5. Birchall, R., Taylor, R. D., Lowenstein, B. E., and Page, I. H.: Clinical Studies of the Pharmacologic Effects of Tetraethyl Ammonium Chloride in Hypertensive Persons Made in an Attempt to Select Patients Suitable for Lumbodorsal Sympathectomy and Ganglionectomy, *Am. J. M. Sc.* 213: 572-578, 1947.

# LABORATORY METHODS

## AN APPARATUS FOR CONTINUOUS RECORDING OF THE VOLUME OF EXPIRED AIR

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VARIOUS methods are available for recording the respiratory minute volume, most common of which is the collection of expired air in a spirometer or Douglas bag. Obviously there are a number of limitations to such a procedure. We therefore sought an apparatus which would give a continuous record, provide exact information from minute to minute, require practically no attention, and be simply and sturdily constructed. Such a unit with these characteristics has been in use in these laboratories for the past three years. It has been especially valuable in quantitative studies comparing respiratory stimulation<sup>1</sup> and depression<sup>2</sup> by drugs. Both man and dog have been employed with the same machine. Although not yet tried, respiration of unanesthetized dogs could easily be measured by utilizing an appropriate mask. A very brief description of the method has been given previously.<sup>3</sup> Details of construction are herein reported.

### APPARATUS

The design of the collecting apparatus is shown in Fig. 1. It consists of two Krogh-type spirometers attached by an overhead cord and pulleys to counter-balance each other. These spirometers are connected to standard one-half inch galvanized pipes to carry the expired air. Four solenoid valves are located in the pipes, two in front of and two behind each spirometer. The solenoids are closed except when activated. The circuit is so arranged that one front valve and the opposite rear solenoid are opened simultaneously, and vice versa. This enables one spirometer to empty while the other is being filled with expired air. Mercury switches are situated above the tanks, contact with which controls the operation of the solenoids. Once contact is made, the mercury switch is inactivated until the tank trips the opposite one. This prevents any possible error, since connections are made to an automatic counter and to a signal magnet for recording on paper. The diagram of the wiring is given in Fig. 2.

In close proximity to the subject or test animal are two valves. Their construction is seen in Fig. 3 and resembles that described by Wright.<sup>4</sup> Each valve consists of a glass tube surrounded by a small pool of mercury on which rests a lightweight inverted glass cup. One valve allows air to enter for inspiration, while the other transmits expired air to the collecting device. The dead air space is small to prevent undesired effects on normal respiration. All tubing is at least 13 mm. inside diameter to allow air to pass freely. The valves operate easily and are of sufficient size to eliminate points of resistance. We have tried

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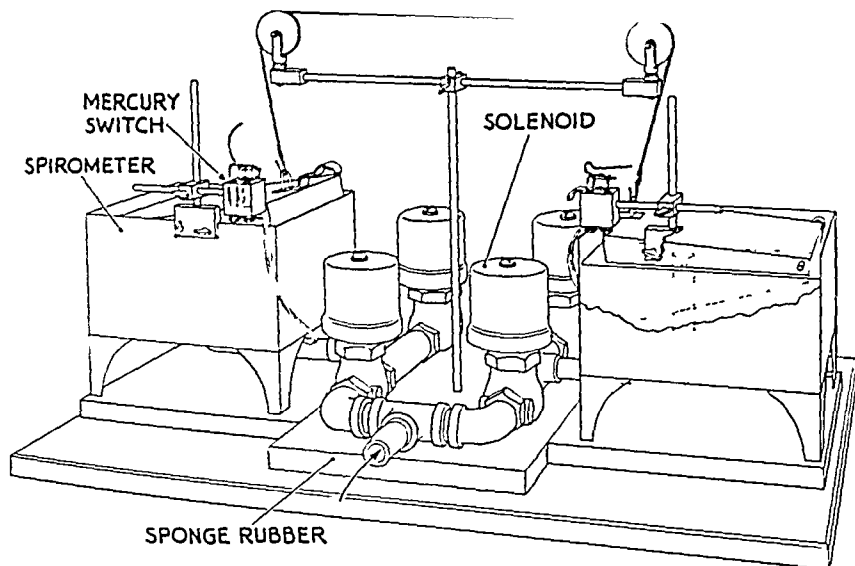


Fig. 1.—Apparatus for continuously collecting and measuring the volume of expired air.

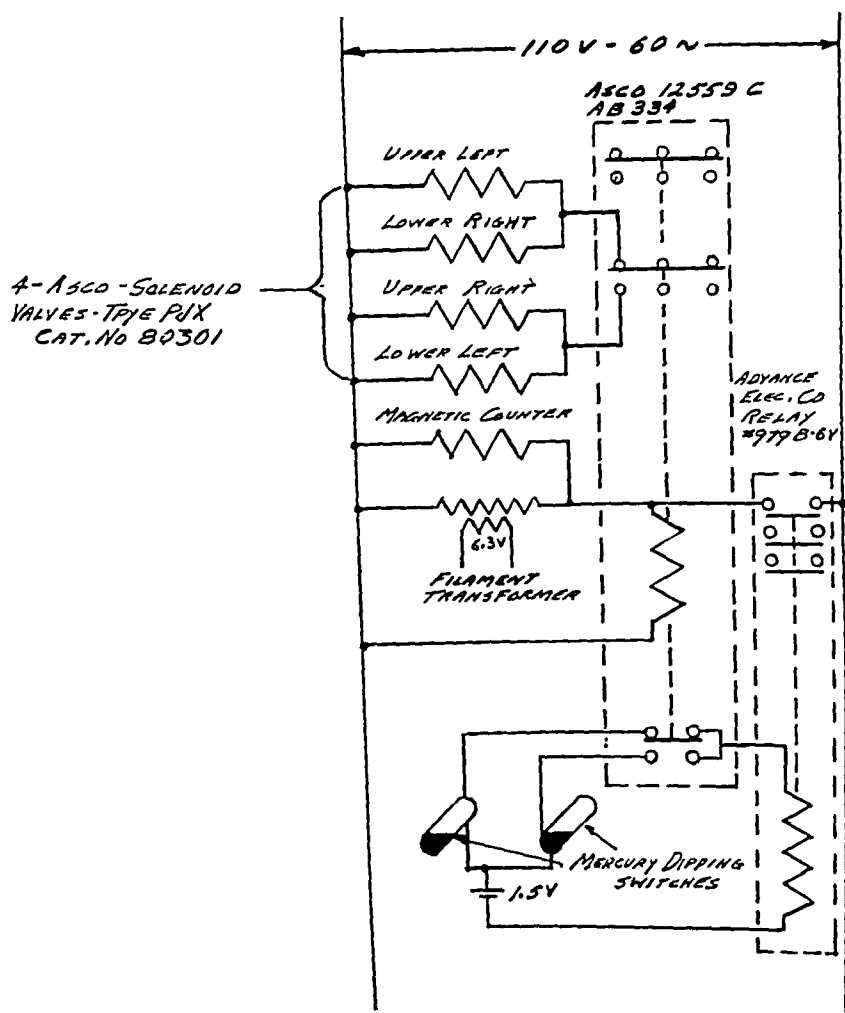


Fig. 2—Wiring diagram of collecting apparatus, switch box, and countersystem

various rubber-flap valves, including those described by Chambers and associates,<sup>5</sup> but in our experience they tend to leak, particularly during strong respiratory movements. A light bulb with reflector placed close to the expiratory valve provides sufficient heat to prevent condensation of moisture.

The exact resistance to flow of air was measured for different rates and depths of respiration. To do this, a water manometer was connected to a side arm of the tube leading to the subject. For comparison, measurements were made also with a standard basal metabolism machine and a 177-liter Tissot spirometer. The results with the three types of apparatus were quite comparable.

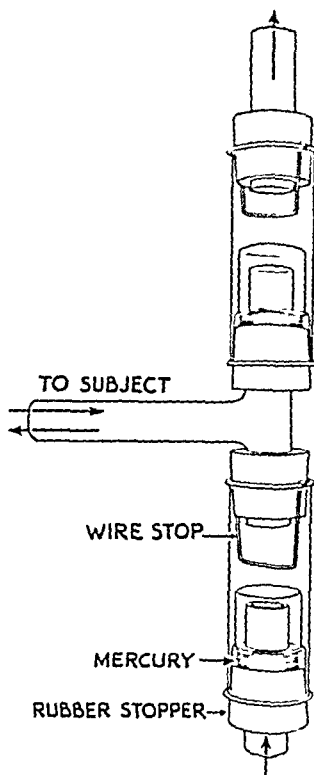


Fig. 3.—Respiration valves which direct expired air to collecting apparatus but prevent re-breathing.

With normal respiration, our machine showed values of  $-5$  mm. water and  $+25$  mm. water on inspiration and expiration, respectively. This compared with  $-7$  mm. and  $+15$  mm. for the Tissot spirometer, and  $-3$  mm. and  $+2$  mm. for the basal metabolism apparatus. When the rate and depth of respiration were augmented, resistance increased greatly with all three devices. Expiratory pressures increased to 70 to 90 mm. water, while there was a decrease of inspiratory pressures to  $-30$  to  $-60$  mm. water. There was no difference between the three methods.

Calibration of the spirometers may be done by a 100 c.c. syringe connected to the animal side of the inspiratory-expiratory valves. By adjusting the

length of the overhead cord, the capacity of the spirometers may be varied. The capacity of the two spirometers is made identical by proper location of the mercury switches. In our experience, 500 c.c. volume changes usually have been employed. Calibration values were determined using different rates of pulsation with the syringe. At rates of 30 to 60 pumps per minute, the error proved to be less than 1 per cent. For instance, ten fillings of each spirometer gave values of  $764.5 \pm 3.91$  c.c. for the left spirometer and  $755.5 \pm 3.98$  c.c. for the right tank, at a rate of 50 pumps per minute. At extremely slow rates of air flow, leakage does occur in the inspiratory-expiratory valve system. This is equally true with rubber-flap valves. However, these rates of air flow have no counterpart in the respiration of human beings or of dogs.

#### METHOD

Human subjects use a mouthpiece such as supplied with the usual metabolism apparatus. The mouthpiece is connected to the inspiratory-expiratory valves which in turn are attached to the spirometers. About five minutes are usually required for the subject or patient to become accustomed to the procedure and for respiration to stabilize. Subsequently, a recording or measuring period of ten minutes is sufficient, but a much longer duration is easily tolerated. This procedure may be repeated at regular intervals to test the prolonged action of such drugs as morphine.

In our experience, sodium phenobarbital is a good anesthetic for respiratory studies in dogs. Owing to its long action, respiration will remain unchanged for hours. This is a distinct advantage in measuring the stimulating or depressing action of any drug.

This apparatus should be easily adaptable to other types of respiratory studies. In case inspired gases other than ordinary air were needed, it would be necessary only to connect a supply of the desired gas or mixture of gases to the tube on the inspiratory valve.

#### SUMMARY

An apparatus is described which continuously records the volume of expired air. Essentially it consists of two spirometers with appropriate valves. While one spirometer fills with expired air, the other tank empties. At each filling, registration is made by both a signal magnet and an automatic counter.

#### REFERENCES

1. Scott, Charles C., Anderson, Robert, C., and Chen, K. K.: Further Study of Some 1-Substituted Theobromine Compounds, *J. Pharmacol. & Exper. Therap.* 86: 113, 1946.
2. Scott, Charles C., and Chen, K. K.: The Action of 1,1-Diphenyl-1-(Dimethyl-aminoisopropyl)-Butanone-2, a Potent Analgesic Agent, *J. Pharmacol. & Exper. Therap.* 87: 63, 1946.
3. Scott, Charles C., and Chen, K. K.: Comparison of the Action of 1-Ethyl Theobromine and Caffeine in Animals and Man, *J. Pharmacol. & Exper. Therap.* 82: 89, 1944.
4. Wright, S.: A Quantitative Graphic Respiration Recorder for Small Animals, *J. Physiol.* 80: 28, 1934.
5. Chambers, A. H., Brewer, G., Davenport, H. W., and Goldschmidt, S.: Respiratory Mask and Valves for Dogs, *Proc. Soc. Exper. Biol. & Med.* 61: 41, 1946.

# A MICROMETHOD FOR THE DETERMINATION OF THE ALBUMIN-GLOBULIN RATIO IN GUINEA PIG SERUM

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WHILE conducting certain studies on the sera of guinea pigs it was found desirable to determine the albumin-globulin ratio of these sera. Since the methods employed generally require the withdrawal by cardiac puncture of considerable amounts of blood, a micromethod was developed requiring only one-tenth of a milliliter of serum. The blood was obtained by making an incision perpendicular to and across the marginal vein of the ear and gently expressing the blood into a capillary tube. The technique of bleeding is described by Ecker, Hiatt, and Barr.<sup>1</sup>

## METHODS

The micromethod is based on the methanol precipitation of the globulins in human serum as employed by Pillemer and Hutchinson.<sup>2</sup> The conditions were standardized in such a manner that the results obtained by the micromethanol precipitation technique agreed with those secured by the electrophoretic analysis of the same sera.

It was noted that at 0 to -5° C. the serum albumin remained soluble at a concentration of 42.5 per cent methanol, pH range of 5.3 to 5.5 (before addition of methanol), and ionic strength of 0.015. Under these conditions all the globulins were precipitated.

TABLE I. COMPARISON OF THE ALBUMIN-GLOBULIN RATIOS OF GUINEA PIG SERA BY ELECTROPHORETIC ANALYSIS AND BY METHANOL PRECIPITATION

	GUINEA PIG*									
	1	2	3	4	5	6	7	8	9	10
Electrophoresis										
Gm. albumin per 100 c.c.	3.15	2.64	2.86	2.96	3.42	3.24	2.39	1.98	2.36	2.58
Gm. globulin per 100 c.c.	3.02	2.95	2.70	3.11	3.19	2.60	2.72	2.47	2.98	1.87
Albumin-globulin ratio	1.04	.89	1.06	.95	1.07	1.25	.88	.80	.79	1.38
A/G Micromethod										
Gm. albumin per 100 c.c.	3.17	2.62	2.69	2.89	3.35	3.25	2.36	1.90	2.44	2.49
Gm. globulin per 100 c.c.	3.00	2.97	2.87	3.18	3.26	2.59	2.75	2.55	2.90	1.96
Albumin-globulin ratio	1.05	.88	.94	.91	1.03	1.26	.86	.74	.84	1.27
Per cent deviation of AG ratio	.96	1.12	11.32	4.21	3.73	.80	2.27	7.50	6.34	7.60
Average deviation, ±6%										

\*Guinea pigs 1 through 6, normal guinea pigs; 7 and 8, bled by cardiac puncture for three successive days before getting serum sample; 9, post-partum guinea pig; 10, 4-week-old guinea pig.

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A comparison of the albumin-globulin ratios obtained electrophoretically and by the micromethod is found in Table I.

The results obtained indicate that the two methods agree closely, the average deviation being less than 5 per cent.

As an additional proof of the value of the micromethod, the globulins of 8 ml. of pooled guinea pig sera were precipitated at the same dilution and under the same conditions as employed in the microtechnique. The globulin precipitate was washed once and dissolved in a veronal buffer (pH, 8.6;  $\mu$ , 0.1) for electrophoretic analysis. The supernatant albumin was lyophilized to dryness and also dissolved in veronal buffer.

The patterns of the two samples showed that the albumin fraction contained less than 2 per cent globulins, while the globulin fraction contained 7.7 per cent of albumin, most of which can be attributed to occlusion.

#### MATERIALS

*Methanol Reagent.*—Chemically pure methanol, 607 ml., are added with mixing to 393 ml. of distilled water. This mixture is cooled to 0° C. and made up to 1 liter with cold methanol.

*Acetate Buffer* (pH, 5.4;  $\mu$ , 0.05).—Sodium acetate  $3\text{H}_2\text{O}$ , 6.8 Gm., and 1 N acetic acid, 10.6 ml., are diluted to 1 liter with distilled water. The pH of the solution should be between 5.3 and 5.5.

#### PROCEDURE

1. Place 2.9 ml. of acetate buffer in a 5 ml. volumetric flask and to this buffer add 0.1 ml. of the test serum with a volumetric pipette.

2. With a volumetric pipette remove 2 ml. of this 1:30 serum and transfer it to a 15 ml. conical centrifuge tube. Blow the small amount remaining in the tip of the pipette back into the 5 ml. flask so that exactly 1 ml. is left.

3. Add 1 ml. of the acetate buffer to the centrifuge tube and fill the volumetric flask to the 5 ml. mark with the buffer.

4. Cool the buffered serum in the centrifuge tube to 1° C. Add 7 ml. of the methanol reagent cooled to 0 to -5° C. Stir well, stopper, and allow to stand for thirty minutes at 0 to -5° C. During this period the globulins will precipitate, leaving the albumin in solution. The suspension is then centrifuged for thirty minutes at 2,000 revolutions per minute in a refrigerated centrifuge at 0 to -5° C.

5. Withdraw a 4 ml. aliquot sample of the supernatant with a volumetric pipette, transfer it to a digestion flask, and determine the nitrogen content.

6. A 4 ml. aliquot sample from the 5 ml. volumetric flask is then withdrawn and transferred to a digestion flask for a determination of its nitrogen content. From this analysis the total serum protein content is obtained. Nitrogen determinations are made according to the Pregl and Grant<sup>4</sup> technique with two blanks, one of which is made up of the same proportions as the albumin supernatant (3 parts buffer and 7 parts methanol), and the other consisting of 4 ml. of buffer.

## CALCULATIONS

$15/4 \times \text{mg. N in supernate} = \text{Gm. albumin per 100 ml. of serum}$   
 $15/4 \times \text{mg. N in total protein sample} = \text{Gm. of total protein per 100 ml. of serum}$

$$\frac{\text{Gm. albumin N per 100 ml. of serum}}{\text{Gm. total protein N per 100 ml. of serum}} = \text{Per cent albumin}$$

$\text{Gm. total protein N per 100 ml. of serum} - \text{Gm. albumin N per 100 ml. of serum} = \text{Gm. globulin N per 100 ml. of serum}$   
 $100 \text{ per cent} - \text{Per cent albumin} = \text{Per cent globulin}$

$$\frac{\text{Per cent albumin}}{\text{Per cent globulin}} = \text{Albumin-globulin ratio.}$$

## SUMMARY

A method is presented which gives the albumin-globulin ratio in as small a quantity as 0.1 ml. of guinea pig serum. Values obtained by this technique compare favorably with those obtained electrophoretically. The conditions found here apply to guinea pig serum. Extension of the method to the sera of man and of other animals requires a readjustment of the conditions for precipitation.

## REFERENCES

1. Ecker, E. E., Hiatt, C. W., and Barr, L. M.: Effect of Pregnancy on the Complement of Guinea Pigs, *J. LAB. & CLIN. MED.* 32: 287, 1947.
2. Pillemer, L., and Hutchinson, M. C.: The Determination of the Albumin and Globulin Contents of Human Serum by Methanol Precipitation, *J. Biol. Chem.* 158: 299, 1945.
3. Pregl, F., and Grant, J.: Quantitative Organic Microanalysis, ed. 4, Philadelphia, 1946, The Blakiston Company.

# SCHLESINGER'S TEST FOR UROBILIN IN THE PRESENCE OF RIBOFLAVIN AND OTHER FLUORESCENT COMPOUNDS

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IN THE past, urinary fluorescence has been noted occasionally following the ingestion of acriflavine,<sup>3</sup> eosin,<sup>13</sup> and fluorescein.<sup>5</sup> With the recent widespread use of vitamin preparations green urinary fluorescence due to riboflavin is often found which interferes with the green fluorescence of urobilin produced in Schlesinger's zinc acetate-alcohol test. Such interference may be troublesome in cases of hepatic diseases in which urobilinuria is an important sign and, at the same time, vitamin B medication is an accepted treatment.

Ehrlich's test for urobilinogen, as recommended by Watson,<sup>20-22</sup> is generally preferred and is not influenced by fluorescent compounds with the exception of acriflavine.<sup>15</sup> The orange color or precipitate of the latter can be prevented by the addition of sodium acetate, according to Watson. Schlesinger's test, however, still has its merits, namely, in cases of old urine specimens where urobilinogen has been oxidized to urobilin, in determinations on urine of jaundiced patients where biliverdin formed by the hydrochloric acid of Ehrlich's reagent may mask the red urobilinogen reaction, in cases of albuminuria where protein interferes with Ehrlich's test,<sup>16</sup> and whenever a confirmation of Ehrlich's test is desired.

The following methods allow the performance of Schlesinger's test in the presence of riboflavin and other dyes exhibiting green fluorescence which may occur in urine, and tests for their identification are given.

## TECHNIQUE OF PROCEDURES

### *I. Schlesinger's Test in the Presence of Riboflavin\**

*Principle.*—Urobilin is extracted with chloroform in which riboflavin is insoluble and the chloroform extract treated with zinc acetate and alcohol.

*Method.*—Ten cubic centimeters of urine, 10 c.c. of chloroform, 10 drops of concentrated HCl, and 2 drops of tincture of iodine are shaken and centrifuged. The chloroform layer is drawn off, mixed with half its volume of alcohol, about 0.2 Gm. of zinc acetate, and 1 drop of concentrated NH<sub>4</sub>OH, and filtered. The filtrate is viewed through the height of the fluid column with strong side light against a background of black velvet for the presence of green fluorescence.

*Comment.*—The extraction of urobilin is not complete. Bilirubin is partially extracted and may exhibit a reddish fluorescence as first observed by Auché<sup>2</sup> and later investigated by Dhéré.<sup>4</sup> This red fluorescence, however, is generally too weak to interfere.

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\*The products used were: riboflavin, Harris Laboratories, St. Louis, Mo.; acriflavine, Abbott Laboratories, North Chicago, Ill.; fluorescein, Merck & Co., Inc., Rahway, N. J.; eosin and erythrosin, National Aniline and Chemical Co., New York, N. Y.; mercurochrome, Hynson, Westcott & Dunning, Inc., Baltimore, Md.

## II. Schlesinger's Test in the Presence of Acriflavine\*

*Principle.*—Acriflavine is rendered insoluble in chloroform by treatment with  $\text{NaNO}_2$  in acid solution, resulting in a purple color reaction.

*Method.*—Ten cubic centimeters of urine are treated with 10 drops of concentrated  $\text{HCl}$  and 10 drops of 5 per cent  $\text{NaNO}_2$  and the test completed as under I.

*Comment.*—An investigation into the nature of the purple color reaction which may be due to formation of a nitroso compound or to simple oxidation has not been attempted.

## III. Schlesinger's Test in the Presence of Fluorescein, Eosin, Erythrosin, and Mercurochrome\*

*Principle.*—Urinary pigments are adsorbed on cotton in a Tweed column and urobilin is eluted with ammonia and alcohol.

*Method.*—A glass tube 20 by 1.5 cm. with tapered end (the lower end of a 100 c.c. burette is suitable) is stuffed moderately tightly with absorbent cotton to a height of 18 cm.; 10 c.c. of urine mixed with 10 drops of concentrated  $\text{HCl}$  and 20 drops of bromine water are poured on the column and allowed to soak into the cotton, followed by 1 c.c. of concentrated  $\text{NH}_4\text{OH}$  and 6 c.c. of 95 per cent alcohol. Two cubic centimeters of filtrate are slowly collected; 10 c.c. of alcohol and 3 drops of concentrated  $\text{NH}_4\text{OH}$  are added. Half of this mixture is filtered without and the other half filtered with the addition of about 0.3 Gm. zinc acetate. Fluorescence of the latter filtrate indicates the presence of urobilin, while the former serves as a control and may show a faint fluorescence due to fluorescein derivatives which were not completely retained by the cotton.

*Comment.*—The procedure is based on Arata's test<sup>1</sup> for the artificial coloring of food. Since urobilin is partly adsorbed<sup>10</sup> and incompletely eluted the test is not very sensitive and a weak fluorescence is evidence of increased urobilin. Bilirubin is retained in the cotton and thus separated from urobilin. Eosin is best adsorbed and fluorescein least, necessitating its conversion to eosin by bromine prior to adsorption. In order to insure sufficient retention of these dyes the pinkish or orange color ring appearing on elution should not move further than about two-thirds down the column.

## IV. Spectroscopic Test of Urobilin and Fluorescent Dyes in Urine

### SPECTROSCOPIC DETECTION OF UROBILIN IN THE PRESENCE OF FLUORESCENT DYES.—

*Principle.*—A urinary chloroform extract free of flavins is treated with zinc acetate, alcohol, and acid whereby the spectrum of acid urobilin is produced while fluorescein derivatives do not give any absorption in acid solution.

*Method.*—The procedure is the same as under I. Five cubic centimeters of the final solution are acidified with 3 drops of concentrated  $\text{HCl}$ , filtered, and examined in a suitable layer with a wave length spectroscopet for the absorption of acid urobilin at 504 to 478 millimicrons.

\*See footnote\* on p. 1503.

†The grating spectroscope of Schmidt and Haensch, Berlin, was used.



*Comment.*—In the presence of much urobilin the usual Schlesinger filtrate may be used, but the absorption band is not so sharply defined. The spectroscopic test is far less sensitive than the fluorescence test.

#### SPECTROSCOPIC DETECTION OF FLUORESCENT DYES IN THE PRESENCE OF UROBILIN.—

*Principle.*—The urine is extracted with amyl alcohol, diluted with 95 per cent alcohol, and rendered alkaline. The comparatively weak absorption band of urobilin does not interfere as a rule.

*Method.*—Ten cubic centimeters of urine (or a larger volume) acidified with 4 drops of concentrated HCl are shaken with 5 cubic centimeter of amyl alcohol and centrifuged. The amyl alcohol is drawn off, mixed with an equal volume of 95 per cent alcohol and 3 drops of concentrated  $\text{NH}_4\text{OH}$ ; and diluted with more alcohol if necessary. The solution is examined with the spectroscope for the absorption bands given in Table I.

TABLE I. SCHEME FOR PRELIMINARY IDENTIFICATION OF FLUORESCENT DYES IN URINE

COMPOUNDS	URINE OF YELLOWISH COLOR; STRONG YELLOW-GREEN FLUORESCENCE IN UV LIGHT	URINE OF PINKISH COLOR; DULL MAUVE FLUORESCENCE IN UV LIGHT	REMARKS
Riboflavin	Not extractable with ether or chloroform; spectroscopic: darkening of right side of spectrum up to about 500 $\text{m}\mu$ Purple color reaction with $\text{NaNO}_2$ and acid; spectroscopic: similar to riboflavin Conversion to eosin with bromine and acid; spectroscopic: 512–491 $\text{m}\mu$		
Aceriflavine			
Fluorescein			
Eosin		Adsorption on cotton; spectroscopic: 537–521 $\text{m}\mu$	Filtering acidified urine through cotton in Gooch crucible
Mercurochrome		Test for mercury; spectroscopic: 534–511 $\text{m}\mu$	Gettler's test <sup>6</sup> in urine, concentrated if necessary. <sup>7</sup>
Erythrosin		Weak fluorescence; spectroscopic: 528–502 $\text{m}\mu$	Identification by absorption curve or biologic test. <sup>3</sup>

*Comment.*—The method of Discombe<sup>5</sup> may be used but it requires larger amounts of urine. Urobilin up to concentrations of 200 mg. per cent in terms of urobilinogen did not interfere when the dye concentrations were 1 mg. per cent. As noted from Table I, the absorption bands of eosin, mercurochrome, and erythrosin are very similar and it is doubtful whether spectroscopy alone will be sufficiently accurate for their differentiation.

#### DISCUSSION

The excretion of riboflavin after vitamin B medication depends on the dose of riboflavin, which may vary from 5 to 50 mg. per day,<sup>11</sup> on urinary concentration, and on the excretory capacity of the kidneys. The fluorescence

in concentrated specimens may be noticed easily under ordinary lighting conditions or may be so weak that it can be seen only when the filtered urine is examined with strong side light. But even weak concentrations are revealed instantly in filtered ultraviolet light by the brilliant yellow-green fluorescence which is superimposed on the gray-white fluorescence of normal urine.<sup>13</sup> Since treatment with vitamin B complex is advocated also in psychoses,<sup>19</sup> fluorescent urines are frequently found in patients in mental institutions.

There are only three reports on urinary fluorescence due to compounds other than riboflavin. Goldschmidt<sup>3</sup> found fluorescence caused by acriflavine (trypanflavin) which is listed in *The Dispensatory of the United States of America*<sup>23</sup> and Gutman's *Modern Drug Encyclopedia and Therapeutic Index*<sup>24</sup> as an external and internal disinfectant and, at one time, was used as a gall bladder antiseptic. Schumm<sup>15</sup> observed a fluorescent urine due to eosin, the source of which could not be determined. Discombe<sup>5</sup> found fluorescein in urine after ingestion of a proprietary drug. As fluorescein is used increasingly as a diagnostic aid, for example for the determination of the circulation time,<sup>14</sup> for the examination of the adequacy of peripheral blood vessels,<sup>12</sup> and for visualizing gastric ulcers during gastroscopy,<sup>17</sup> one may expect to find fluorescein in urine more often.

The author noted several cases of urinary fluorescence due to acriflavine following medication, two cases due to mercurochrome subsequent to bladder irrigation, and three cases due to fluorescein of undetermined origin. Recently, an eosinlike dye was found in urine following the swallowing of a lipstick by a mental patient. According to information received from the Food and Drug Administration\* this dye most probably was rhodamine B, commonly used for coloring lipsticks. Another fluorescent urine on extraction yielded a dye which showed some characteristics of erythrosin but could not be identified definitely due to insufficient material. Erythrosin, the only red coal tar dye permitted for coloring food, is presumably ingested frequently but, due to its weak fluorescence and the small quantities excreted, has probably escaped notice.

The scant reports in the literature do not necessarily indicate that urinary fluorescence is not being encountered from time to time by laboratory workers and, if present in Schlesinger filtrates, it may be wrongly interpreted as urobilinuria. For such occasions it was thought helpful to refer to Table I which presents a simple scheme for the preliminary identification of fluorescent dyes which may occur in urine.

#### SUMMARY

1. A modified Schlesinger test has been described allowing the separation of urobilin from riboflavin whose green fluorescence in urine following vitamin B medication interferes with that of the urobilin-zinc compound.

2. Modifications of Schlesinger's test in the presence of fluorescent dyes occasionally present in urine, such as acriflavine and fluorescein and derivatives, have been described.

3. Tests for identification of fluorescent dyes in urine have been given.

\*I wish to express my thanks to G. Robert Clark, Acting Chief, Cosmetic Division, Food and Drug Administration, for his helpful information.

## REFERENCES

1. Arata, P. N.: Cited in Leach, A. E., and Winton, A. L.: *Food Inspection and Analysis*, New York, 1920, John Wiley & Sons, p. 841.
2. Auché, A.: Sur un spectre caractéristique des pigments biliaires, *Compt. Rend. Soc. de biol.* 64: 299, 1908.
3. Conn, H. J., Ambler, J. A., and others: *Biological Stains*, Geneva, N. Y., 1940, Biotech Publications, p. 249.
4. Dhéré, C.: Etude spectrochimique d'un dérivé de la bilirubine possédant une fluorescence rouge, *Arch. internat. de pharmacodyn. et de thérapie* 38: 134, 1930.
5. Discombe, G.: Fluoresceinuria, *Lancet* 1: 86, 1937.
6. Gettler, A. O.: Simple Tests for Mercury in Body Fluids and Tissues, *Am. J. Clin. Path. (Tech. Supp.)* 7: 13, 1937.
7. Gettler, A. O.: Toxicology in General Hospitals, *Am. J. Clin. Path. (Tech. Supp.)* 3: 76, 1939.
8. Goldschmidt, G.: Zur Spezifität der Ehrlichschen Aldehydreaktion im Harn, *Deutsche med. Wchnschr.* 56: 140, 1930.
9. Gutman, A. B.: *Modern Drug Encyclopedia and Therapeutic Index*, New York, 1946, The Yorke Publishing Company, Inc., pp. 11, 314, 453, 455, 631.
10. Hoppe-Seyler, F., and Thierfelder, H.: *Handbuch der physiologisch- und pathologisch-chemischen Analyse*, Berlin, 1924, Julius Springer, p. 430.
11. Ingelfinger, F. J.: The Parenteral Use of Vitamin Preparations, *New England J. Med.* 233: 379, 1945.
12. Moore, G. E., and Campbell, G. S.: Intra-Arterial Fluorescein for Evaluation of Peripheral Vascular Disease, *Proc. Soc. Exper. Biol. & Med.* 65: 261, 1947.
13. Najjar, V. A., and Holt, L. E., Jr.: The Excretion of Specific Fluorescent Substances in the Urine in Pellagra, *Science* 93: 20, 1941.
14. Nathanson, M. H., and Merliss, R.: A Method for the Study of Circulation Time Throughout the Vascular System, *Proc. Soc. Exper. Biol. & Med.* 53: 261, 1947.
15. Naumann, H. N.: The Reaction of Urobilinogen With p-Dimethylaminobenzaldehyde, *J. LAB. & CLIN. MED.* 23: 1127, 1938.
16. Naumann, H. N.: A Study of Ehrlich's Test for Urobilinogen and Schlesinger's Reaction for Urobilin, *Biochem. J.* 30: 347, 1936.
17. Robinson, H. M.: Fluorescein—Aid in Gastroscopy, *Rev. Gastroenterol.* 13: 303, 1946.
18. Schumm, O.: *Spektrochemische Analyse*, Jena, 1927, Gustav Fischer, p. 218-219.
19. Wadsworth, G. L., Quesnel, E., Murphy, E. C., Gerson, M., Fish, V., and Nogee, P.: Evaluation of Treatment of Senile Psychosis With Vitamin B Complex, *Am. J. Psychiat.* 99: 807, 1943.
20. Watson, C. J.: *Bile Pigments*, New England J. Med. 227: 665, 705, 1942.
21. Watson, C. J.: Cirrhosis of the Liver; Clinical Aspects With Particular Reference to Liver Function Tests, *Am. J. Clin. Path.* 14: 129, 1944.
22. Watson, C. J.: The Bedside Diagnosis of Parenchymatous Liver Disease, *Illinois M. J.* 86: 196, 1944.
23. Wood, H. C., and Osol, A.: *The Dispensatory of the United States of America*, Philadelphia, 1943, J. B. Lippincott Company, pp. 61, 477, 681, 939, 1322.

# PROCEEDINGS OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

Twentieth Annual Meeting  
Chicago, Ill., Oct. 31 and Nov. 1, 1947

## TO BE READ BY TITLE—CONCLUDED

### 51. CONTINUOUS OBSERVATIONS OF THE ARTERIAL OXYGEN SATURATION AT REST AND DURING EXERCISE IN CONGENITAL HEART DISEASE

GEORGE E. MONTGOMERY, JR., M.D. (BY INVITATION), EARL H. WOOD, M.D. (BY INVITATION), HOWARD B. BURCHELL, M.D., THOMAS J. DRY, M.D., ROBERT L. PARKER, M.D. (BY INVITATION), AND H. FREDERIC HELMHOLZ, JR., M.D., ROCHESTER, MINN.

Analysis of arterial samples from twenty resting persons with congenital heart disease has been used as a reference point for continuous measurements of the arterial oxygen saturation with the Milliken compensated circuit oximeter during exercise and other procedures. Nineteen normal volunteers were studied as controls. Observations have been made while the individuals breathed pure oxygen, with and without positive pressure up to four inches (10.2 cm.) of water, while they stood upright, and while they walked on a power-driven treadmill at 1.7 miles per hour.

In normal persons at rest, oximeter readings indicated that the arterial saturation was increased by from 1 to 5 percentage points (mean, 2.7) in an average time of 1.3 minutes when pure oxygen was breathed. Addition of positive pressure to the oxygen supply caused no further change in the oximeter reading.

In resting patients with cyanotic types of congenital heart disease the arterial oxygen saturation averaged 71 per cent, ranging from 45 to 91 per cent saturation. Oximeter readings were increased from 2.0 to 16.5 percentage points (mean, 6.2) in an average time of 3.0 minutes when these patients breathed pure oxygen. When the oxygen was given with positive pressure, oximeter readings increased, on the average, an additional 1.6 percentage points.

The normal group showed no significant change in their arterial oxygen saturation when they assumed the upright position or when they exercised by walking on a horizontal treadmill at 1.7 miles per hour for five minutes. The oximeter readings of persons with cyanotic types of congenital cardiac defects decreased, on the average, 2.4 percentage points when they stood upright, and decreases ranging from 3.5 to 19.0 percentage points (mean, 10.9) occurred when they walked on the treadmill at 1.7 miles per hour for an average time of 3.5 minutes.

Simultaneous arterial samples and oximeter readings obtained both at rest and during exercise in a smaller series of patients indicated that the saturation

changes recorded by the oximeter were less than the changes found by the van Slyke analysis of arterial blood. Nevertheless, use of the oximeter in these tests has proved a valuable objective adjunct in assessing the degree of dysfunction in such patients both before and after surgical corrective procedures have been attempted.

## 52. A TWELVE-YEAR STUDY OF A PARTICULAR GROUP OF HYPERTENSIVE PATIENTS PRE- AND POSTSYMPATHECTOMY WITH REFERENCE TO RENAL PATHOLOGY

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(BY INVITATION), CHICAGO, ILL.

In the past twelve years twenty-five cases of essential hypertension were studied jointly by the medical and surgical services of a university hospital. A part of this study is reported in this paper, the correlation of vasomotor tests with the anatomic changes found in renal biopsies (six) or at autopsy (four).

Selection of a particular group of patients for consideration of surgical therapy was made in the hope that eventually the surgical treatment of hypertension might be completely and properly evaluated. These patients were those whose course was rapidly progressive despite vigorous, nonsurgical therapy. The immediate prognosis in all instances was considered to be good; the ultimate prognosis, however, was always poor.

The same surgeon (Dr. Loyal Davis) performed the several types of sympathectomy, adding thereby to the uniformity of the results.

During the period of this study numerous tests for vasomotor lability were introduced as an aid in the selection of cases suitable for sympathectomy. The following tests were employed: (1) resting blood pressure, (2) sodium amytal test, (3) cold pressor test, (4) continuous caudal anesthesia, (5) carbon dioxide sensitivity, (6) reaction to tetraethylammonium chloride, and (7) a new test, the vertavis test. Most were repeated after sympathectomy and some were observed in the interval between operations in cases where a thoracolumbar sympathectomy was performed. These tests have been compared with the anatomic changes found and the clinical course to date. During the period of observation, none of the tests showed a high degree of correlation with reference to these two factors. Some of the patients reported on here have been followed continuously for twelve years after sympathectomy. Proper case selection for the surgical treatment of hypertension, we feel, is still most difficult, if not impossible, at this time. It is our impression that patients not responding to thiocyanate therapy are unsuitable for sympathectomy.

## 53. THE EFFECTS OF A DIET OF RICE AND FRUIT JUICES UPON THE BLOOD PRESSURE OF HYPERSENSITIVE INDIVIDUALS\*

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(INTRODUCED BY EDWARD H. REINHARD, M.D., ST. LOUIS, MO.)

Attempts were made to evaluate the use of a diet composed of rice, fruit juices, and vitamin supplements in the treatment of arterial hypertension, since others have reported beneficial effects from this and other low salt diets.

\*This study was supported by a grant-in-aid from the United States Public Health Service.

Six patients, five of whom showed essentially normal renal function, were studied extensively, control periods of approximately one month in the hospital being observed. These patients suffered from arterial hypertension in varying degrees of severity, two being in the malignant stage and two in severe and two in moderately severe stages. Patients were placed on the diet for three to five weeks.

In four patients the average diastolic blood pressure changed less than 10 mm. Hg. In the other two it fell 11 and 13 mm. Hg while on the diet but did not return to the control level when a normal diet was substituted.

An attempt was made to determine whether the limitation of salt intake imposed by the special diet played a part in the observed effects upon blood pressure. Serum chloride levels fell in all cases, but the serum sodium concentration did not. Addition of sodium chloride to the diet produced no consistent effects upon blood pressure. Weight was lost by four patients.

It is concluded that the effects of the rice and fruit juice diet upon blood pressure were minimal and inconsequential. Effects which were observed could not be correlated with changes in the salt content of the diet.

#### 54. FURTHER STUDIES WITH THE HISTAMINE TEST FOR PHEOCHROMOCYTOMA

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Previously we reported on the intravenous injection of small amounts of histamine base as a test for distinguishing the syndrome of pheochromocytoma from other clinical conditions. At that time, the injection of 0.05 or 0.025 mg. of histamine base produced the clinical syndrome, including hypertension and the characteristic symptoms, in three patients known to have pheochromocytoma. After surgical removal of the tumor, the injection of the same amount of histamine produced no paroxysm of hypertension or symptoms. As a result, we tentatively suggested this method as a test for the clinical syndrome of pheochromocytoma.

We now wish to present two additional cases of pheochromocytoma. In one of these, paroxysmal hypertension was present and the result of the histamine test was positive. The test was of proved value in arrival at the diagnosis. In the second patient, persistent hypertension with edema of the optic discs was present. The result of the histamine test in this case was regarded as negative because the increase in blood pressure after the injection of histamine did not exceed the increase in blood pressure during the cold pressor test. The failure to secure a positive result in this case of persistent hypertension may possibly be explained on the basis that the tumor contained only a relatively small amount of epinephrine (62 mg.) and that it was probably incapable of secreting sudden explosive charges of epinephrine because it was secreting epinephrine continuously.

A total of four cases, however, can now be reported in which the injection of histamine into patients with pheochromocytoma will produce an "attack" exactly similar to that of which they complain, in which marked elevation of the blood pressure occurs.

Tetraethylammonium chloride failed to produce a paroxysm of hypertension on two occasions in the patient known to have a pheochromocytoma.

The histamine test has been carried out on 200 patients with hypertension and fifty other patients with other clinical conditions. No untoward effects have been experienced by any of these patients. This would seem to indicate that the test is not hazardous.

## 55. DIRECT MEASUREMENT OF BLOOD PRESSURE IN THE RADIAL AND FEMORAL ARTERIES DURING AND AFTER TILTING

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JOHNSON MCGUIRE, M.D., CINCINNATI, OHIO

Direct tracings of radial and femoral artery pressure were obtained by a Satham Strain Gauge during and after tilting at a moderate rate between the 20 degree erect and 45 degree head-down position. The recordings from the radial artery were made with the arm maintained in a horizontal plane throughout the tilt.

Blood pressure in the radial artery invariably rose during the tilt from the head-up to the head-down position. In normal subjects, this increase of blood pressure (average, 19 mm. systolic/14 mm. diastolic) was followed by a fall, the blood pressure returning within eight to eighteen seconds to approximately the level of the head-up position. During the return tilt to the head-up position, the blood pressure always fell. In normal subjects, the pressure decreased (average, 14 mm. systolic/13 mm. diastolic) to a level less than in the initial erect position and returned within eight to eighteen seconds to approximately the initial level in the erect position.

The blood pressure in the femoral artery fell during the tilt from the head-up to the head-down position, and in the normal subjects then fell further, reaching a stabilized level in eight to eighteen seconds. During the return to the head-up position, the femoral blood pressure always increased, and in normal subjects this increase was followed by a further rise, the blood pressure reaching the range of the starting level in eight to eighteen seconds.

The increase in radial artery pressure and the decrease in femoral artery pressure during the tilt from the erect to the head-down position and the opposite changes during the return tilt indicated that these alterations are secondary to gravity. Since these primary changes were followed in both arteries by a fall of blood pressure in the head-down position and by an elevation of blood pressure in the erect position, it is believed that these secondary responses were neurogenic. The changes in the radial artery are interpreted as a reflection of the changes in the large arteries arising from the aortic arch, since the arm was maintained at the level of the arch throughout the tilt. The neurogenic responses tended to maintain the blood pressure at a stable level at the region of the aortic arch. This suggests that the purpose of these reflex adjustments of blood pressure is to protect the brain and perhaps the heart from adverse pressure changes.

## 56. CLINICAL AND EXPERIMENTAL STUDIES ON THE EFFECTS OF DIATHERMY ON BLOOD FLOW

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JEROME GERSTEN, M.D. (BY INVITATION), ROCHESTER, MINN.

In normal individuals as well as in patients (none of whom had any impairment in circulation), the blood flow in the upper and lower extremities was studied by the use of the plethysmograph with a compensating spirometer recorder before and for thirty to thirty-five minutes after application of diathermy for half an hour. Within five to fifteen minutes after diathermy, in seven of the twenty-five patients, the blood flow in the treated extremity increased more than 100 per cent. The increase in blood flow in the twenty-five patients ranged from 39 to 268 per cent, with an average increase of 71 per cent. At the same time the untreated extremities showed an average increase of 5 per

cent, but in nine of the twenty-five patients the untreated extremity showed an initial decrease in blood flow. At the end of thirty minutes after stopping the diathermy treatment, the blood flow in the treated extremities still showed an average increase of 56 per cent over the control value. There was no significant difference between the results obtained on patients and on normal individuals.

In dogs anesthetized with pentobarbital sodium, the blood flow was studied, by means of a bubble flowmeter in the femoral artery or vein, before, during, and after application of diathermy to the thigh for a period of twenty minutes. During the application of diathermy the significant increases over the control blood flow ranged from 16 to 209 per cent, with an average increase of 69 per cent. In four of the seventeen experiments slight decreases of 24 per cent, 18 per cent, 5 per cent, and 5 per cent, respectively, from the control blood flow occurred. After treatment with diathermy was stopped, the significant increases in blood flow ranged from 16 to 142 per cent, with an average increase of 64 per cent. In two of the seventeen experiments there was a slight decrease in blood flow after treatment with diathermy was stopped; namely, 15 and 10 per cent, respectively. In one experiment blood flow did not change after cessation of diathermy. Subcutaneous and muscle temperatures were taken in the dog by means of needle thermocouples and were recorded galvanometrically. The increase in muscle temperature at the end of the application of diathermy ranged from 0.9 to 9° C., with an average increase of 4.0° C.

#### 57. A QUANTITATIVE HYPOTHERMAL METHOD FOR PRODUCTION OF LOCAL INJURY TO TISSUE

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Local necrosis of tissue can be produced rapidly and quantitatively by use of an instrument cooled to a low temperature by internal expansion of carbon dioxide or flow of cold liquids. The cooling element of the instrument is flat and circular, so that when it is applied to the surface of an organ or tissue, cylindrical lesions are produced. The diameters of the lesions can be varied from 2 to 25 mm. by using cooling elements of comparable diameters. The depths of lesions can be varied from 1 to 13 mm. by varying the time of contact of the cooling element with the tissue. Volumes of injured tissue can be accurately determined by measurement. Lesions of identical size and location may be reproduced in successive experiments. Lesions have been produced in cartilage, bone, skin, skeletal muscle, vascular walls, heart, liver, kidneys, and brain of rabbits. Necrosis is uniform throughout the lesions and there is a sharp line of demarcation between nonviable and normal tissue at the periphery. Suppuration does not occur and hemorrhage is never found except around blood vessels in lesions of the brain. Organs and tissues can be progressively and selectively destroyed in a manner which is not possible by other methods such as cauterization, vascular ligation, or surgical excision.



### 38. ANEURYSMS OF THE CORONARY ARTERIES DUE TO POLY-ARTERITIS NODOSA OCCURRING IN AN INFANT: REPORT OF A CASE WITH CORONARY ARTERY THROMBOSIS AND MYOCARDIAL INFARCTION

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(INTRODUCED BY W. B. WOOD, JR., M.D.)

Since polyarteritis nodosa occurs infrequently in infancy and aneurysms of the coronary arteries with coronary artery thrombosis and myocardial infarction represent a rare complication, the following instance involving a 15-month-old infant is reported.

The patient, a white female infant, had been well until the age of 1 year, at which time because of rash and fever of three days' duration the patient was admitted to the Children's Hospital, St. Louis. The infant appeared acutely but not critically ill. The temperature was 39° C. In addition to the rash which was believed to represent an erythema multiforme, there was marked conjunctivitis and pharyngitis. The tympanic membranes were edematous. The remainder of the physical examination revealed nothing pertinent. The blood showed a leucocyte count of 16,000 with normal differential and a hemoglobin of 9 Gm. per cent. Tuberculin and Kline tests were negative and no pathogens were identified in urine, stool, and throat cultures. The rash began to fade on the fourth hospital day but the infant continued to appear ill. The temperature remained between 38 and 40° C. until the tenth day and then slowly subsided. The patient was discharged as improved on the seventeenth hospital day.

Three months later, on Sept. 16, 1946, the patient was readmitted because of dyspnea, orthopnea, anorexia, and fatigue on slight exertion of three weeks' duration. She appeared acutely and chronically ill. The heart and respiratory rates were rapid and the heart sounds of poor quality. Cardiac enlargement and pulmonary congestion were demonstrated by fluoroscopy. Blood examination revealed a leucocyte count of 10,400 per cubic millimeter, a moderate lymphocytosis, and hemoglobin of 10 Gm. per cent. Several hours after admission the infant suddenly became rigid, made some convulsive movements, and ceased to breathe. Emergency therapy revived the patient only temporarily and she expired five hours after admission.

At autopsy the heart presented the most significant and interesting findings. It weighed 80 grams (normal, 50 grams). The left coronary artery had a normal ostium but the lumen was narrowed to pin-point size at the bifurcation. The anterior descending branch of the left coronary artery presented a fusiform enlargement which began 3 mm. from its origin and extended for a distance of 15 millimeters. It measured 10 mm. in its greatest diameter. An adherent thrombus measuring 30 mm. and completely occluding the lumen was found in this branch of the left coronary artery. A portion of the thrombus occluded the circumflex branch at its origin. For most of its course the circumflex branch had a thickened wall and narrowed lumen. Fifteen millimeters from its origin the right coronary artery presented an aneurysmal dilatation measuring 18 mm. in length and 6 mm. in diameter. The wall of the artery was thickened for the initial 35 mm. of its course.

The left ventricle was dilated and the wall thickened (8 mm.) at the base but thin (3 mm.) at the apex. There were evidences of recent and remote infarction in the anterior portion of the interventricular septum and the anterior and apical portions of the left ventricle. The right ventricular wall was hypertrophied (6 mm.). Microscopically vascular changes representing healing and

healed polyarteritis nodosa were observed. Diffuse fibrosis and numerous areas of fresh infarction were found in sections of the anterior and apical portions of the myocardium of the left ventricle and the anterior portion of the septum.

## 59. ORAL MERCURIAL DIURETICS IN AMBULATORY PATIENTS WITH CONGESTIVE HEART FAILURE

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(INTRODUCED BY JOHNSON MCGUIRE, M.D.)

Previous reports of the use of oral mercurial diuretics in the treatment of the decompensated cardiac patient have stressed their use during hospitalization. We are presenting a preliminary report of the use of orally administered mercurial diuretics upon ambulatory patients seen in the Cardiac Clinic of the Out-Patient Dispensary at the Cincinnati General Hospital.

Fifty-six patients with cardiac edema have been treated from three to six months with orally administered mercurial diuretics, using Mercuzanthin.\* All patients had previously received mercurials parenterally. The type of heart disease in twenty-six was hypertension; in fourteen, rheumatic; in eight, arteriosclerotic; in seven syphilitic; and in one, congenital.

The dosage schedule has varied, but a majority of patients now under treatment are taking two or three tablets daily. A minority take three tablets two or three times weekly.

To determine the adequacy of renal function, measurement of the blood urea nitrogen and a complete urinalysis were performed on each patient prior to initiation of therapy. Repeat blood urea nitrogen determinations were performed monthly, and urinalysis was done bimonthly.

Clinical improvement was gauged objectively by weight loss, decrease of edema and râles, increase in vital capacity, decrease in venous pressure; and subjectively by decrease in effort dyspnea, orthopnea, and paroxysmal nocturnal dyspnea.

Of the fifty-six patients treated, forty are currently receiving therapy. Thirty-five are considered improved and five unimproved. In nine patients the treatment has been discontinued for various reasons; these include four instances of painful stomatitis and two of diarrhea. Seven patients have died during the course of therapy; however, a review of the clinical records and autopsy protocols does not suggest that death was the result of mercurial toxicity. No renal complications have been observed. Stomatitis has appeared in eight patients, sufficiently severe to require discontinuance of therapy in four. Abdominal symptoms consisting of nausea, vomiting, abdominal cramps, and diarrhea have appeared in eight patients; treatment was stopped in three. Therapy was later restarted and continued successfully in one of these.

Our conclusion is that oral mercurial diuretics frequently can effectively replace parenteral mercurials in the ambulatory patient and that such replacement has materially reduced the daily patient load in this Cardiac Clinic.

\*Mercuzanthin (Distributed by Campbell Products, Inc., New York, N. Y. Each tablet is equivalent to 0.030 Gm. mercury and 0.027 Gm. anhydrous theophylline).

# 60. PHYSIOLOGIC ADJUSTMENTS TO SUDDEN CHANGES IN ENVIRONMENTAL TEMPERATURE AND HUMIDITY OF HEALTHY YOUNG SUBJECTS AND CARDIAC PATIENTS

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E. TELSER, M.D. (BY INVITATION), IRWIN R. CALLEN, M.D.  
(BY INVITATION), AND CHARLES A. GIANASI, M.D.  
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Physiologic adjustments of healthy young male medical students to sudden changes in environmental temperature and humidity have been studied. The subjects remained for one hour in a comfortable environment (76° F. with relative humidities of 30, 60, or 80 per cent) and then entered a hot moist environment of (98.5° and 66 per cent relative humidity) for one or two hours. Then they re-entered the comfortable room and remained for one hour.

When subjects entered a hot moist environment from a comfortable environment, their mean skin temperature rose quickly and approximated its maximum value in less than ten minutes. The rectal temperature decreased with the first ten minutes and then slowly increased. The pulse rate and systolic pressure increased slightly while the diastolic pressure decreased slightly.

When they entered an environment conditioned for comfort from a hot moist environment, the mean skin temperature fell rapidly but it fell less rapidly when the relative humidity was high (80 per cent). The rectal temperature increased within the first ten minutes and then slowly decreased. The blood pressure showed no significant variation, although large fluctuations were occasionally observed. The pulse rate decreased but it decreased less when the relative humidity was high (80 per cent). In addition, the subjective sensation of comfort was obtained earlier and the evaporative weight loss per gram of moisture present on the skin and in the union suit was less when the relative humidity was high.

Patients with cardiovascular impairment (arteriosclerotic heart disease, angina pectoris following coronary infarction, hypertensive heart disease, rheumatic heart) are now being studied under comparable experimental conditions. In addition to the observations mentioned, determination of plasma volume, vital capacity, basal metabolic rate, and the effects of Master's two-step tests were made to define the degree of circulation impairment. Preliminary data on these impaired older patients reveal that their thermal adjustments are accomplished with equal ease and no clinically significant greater strain on the cardiovascular system occurs than that shown by the healthy young medical students.

## 61. A CARDIAC FACTOR WHICH INCREASES CORONARY FLOW UNDER SYMPATHETIC STIMULATION

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W. H. PRITCHARD AND C. V. DOWLING† (BY INVITATION), CLEVELAND, OHIO

Stimulation of the stellate ganglion and cardiac nerves was shown by Gregg and Shipley to increase coronary blood flow. The exact mechanism of this change remains unknown.

In a further study of this problem in the anesthetized open-chest dog, blood flow in the left anterior descending or left circumflex coronary arteries was

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measured by a recording rotameter and a phasic flow meter either under the normal pulsatile aortic pressure or under a constant perfusion pressure. Flow measurements were made before, during, and after stimulation of the left cardiac accelerator nerves both before and after section from the stellate ganglion.

When both mean and phasic flows were recorded simultaneously, mean flow often increased before the phasic flow meter revealed any change in the resistance to flow as indicated by a constant rate of diastolic flow. Inspection of aortic and intraventricular pressure curves showed a marked shortening of cardiac contraction, as described by Wiggers and Katz, following cardiac nerve stimulation and during the action of adrenalin. In addition to the systolic shortening, our records show a marked increase in the rate of diastolic relaxation.

Actual flow measurements and calculations of increases in absolute lengths of diastole during minute intervals (from intraventricular curves) show that these alterations in systolic and diastolic time ratios account for from 10 to 30 per cent increases in flow, in addition to further increases due to decreased resistance to flow. In this manner, the heart, by its increased velocity of contraction and relaxation, reduces the time of intramyocardial vascular compression during systole, thus substituting longer periods of the high diastolic flow for long periods of low systolic flow despite moderate increases in heart rate. Since the highest rate of coronary blood flow occurs in diastole, it follows that any lengthening of the total diastolic time in a given minute must increase mean blood flow.

These studies are being continued in an effort to evaluate other mechanical factors responsible for increasing coronary flow after nerve stimulation such as changing heart size, intramyocardial tension, and increased vascular emptying resulting from the augmented vigor of contraction.

## 62. THE VENTRICULAR GRADIENT IN HYPERTHYROID CONDITIONS

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(INTRODUCED BY JOHNSON MCGUIRE, M.D.)

In a study of the effect of hyperthyroidism and of thyroid substance on the heart, the electrocardiograms of a group of cases were analyzed by study of their ventricular gradients. These cases were of two groups, the first composed of fourteen patients with hyperthyroidism and the second of eleven normal subjects to whom 6 grains of thyroid substance were administered daily. All patients studied were free of complicating diseases and all had electrocardiograms that were normal except for a few instances where transient abnormalities of cardiac rhythm were noted.

In the group with hyperthyroidism all of the fourteen patients showed an increase in the magnitude of their ventricular gradient as compared with control electrocardiograms taken after remission of the disease induced by surgery or by thiouracil. This increase averaged 39 per cent, with a range from 4 to 100 per cent. In addition, seven of this group showed a significant clockwise shift in the direction of the ventricular gradient averaging 26 degrees, with a range of plus 16 to plus 48 degrees. The other seven in this group showed no significant shift in the direction of the gradient.

In the group receiving thyroid extract, all of the eleven subjects showed an increase in magnitude of the ventricular gradient. This magnitude averaged 30 per cent, with a range from 7 to 73 per cent. None of these subjects showed any significant shift in direction of the gradient.

In both groups increase in heart rate was a common occurrence, and values obtained for magnitude and direction of ventricular gradients were corrected for changes in heart rate.

It is suggested that the increased magnitude of the ventricular gradient is a reflection of the increased work of the heart. It is also suggested that the shift in direction of the gradient in the hyperthyroid group may indicate myocardial strain since this finding has been previously noted in cases of hypertension and of acute glomerular nephritis.

### 63. THE PRECORDIAL ELECTROCARDIOGRAM IN LEFT VENTRICULAR HYPERTROPHY: A STUDY OF AUTOPSIED CASES

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An analysis has been made of the Wilson precordial leads in eighty-four pathologically proved cases of left ventricular hypertrophy without associated myocardial infarction or appreciable gross fibrosis. This comprises all cases occurring within a five-year period in which satisfactory electrocardiograms were obtained within three months of death except for thirty-seven cases of bundle branch block.

By means of a Cambridge device, measurements of the time elapsing from the onset of QRS to the nadir of Q, to peak of R, to nadir of S, and to end of QRS were made in three complexes of Leads  $V_5$ ,  $V_6$ . The average amplitude of the individual components of the QRS, the contour and level of the RST segment, and the amplitude and direction of the T waves were determined in each precordial lead. Similar measurements were made in a control series of fifty-two cases in which the hearts were normal at autopsy, and in a second group of fifty young male adults with normal hearts by physical and roentgen examination.

Differentiation between pattern of left ventricular hypertrophy and of normals could not be made from the amplitude of the R waves in  $V_5$  and  $V_6$  alone since the average values were only slightly higher in the former.

In 98.0 per cent of normals the time interval from the onset of QRS to peak of R was less than .05 second. In 93.1 per cent of normals the time interval from onset of the R wave to its peak was less than .04 second. For purposes of classification, measurements equalling or exceeding these figures were regarded as abnormal. A Q-R of .048-.049 and/or an R of .038-.039 second were regarded as borderline and values below this, as normal. Of the eighty-four patients with left ventricular hypertrophy, thirty-four (or 40.5 per cent) showed an abnormal Q-R and/or R duration. The average cardiac weight in this group was 610 grams. Eight (or 9.5 per cent) showed borderline values and the cardiac weight averaged 569 grams. Forty-two cases (or 50 per cent) showed normal values, with an average cardiac weight of 536 grams. There was a general trend toward increasing duration of Q-R or R with increasing cardiac weight, but many individual exceptions were encountered.

In eight cases where R or RST-T findings in precordial Leads  $V_5$  and  $V_6$  were borderline or normal, abnormalities referable to left ventricular hypertrophy were found either in  $V_7$ , or in the unipolar extremity leads.

In seven cases the electrocardiogram showed nonspecific abnormalities and in four it was regarded as normal.

#### 64. THE FORM OF THE ELECTROCARDIOGRAM IN ANTEROSEPTAL MYOCARDIAL INFARCTION COMPLICATED BY RIGHT AND LEFT BUNDLE BRANCH BLOCK

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Clinical and experimental studies have shown that although the electrocardiographic diagnosis of anterior infarction can be made in the presence of right bundle branch block, the characteristic signs of myocardial infarction are obscured by left bundle branch block unless the infarct involves the interventricular septum. Observations have been made in two patients in whom anteroseptal myocardial infarction was complicated first by transient right bundle branch block and then by transient left bundle branch block. This permitted an unusual opportunity to compare in the same individuals the standard and precordial electrocardiograms in normal conduction and both types of intraventricular block in the presence of myocardial infarction.

The first patient was a man aged 50 years, who had a typical acute coronary thrombosis without antecedent cardiac symptoms. When right bundle branch block was present the standard electrocardiograms showed large S waves in all leads, while records from the right precordium displayed prominent Q waves and large late R deflections. When left branch block was present, Lead I showed no negative deflections and the large Q waves and late R peaks previously recorded from the right, now appeared over the left precordium. The characteristic signs of infarction were confined to Leads CR<sub>2</sub> and CR<sub>3</sub> when normal conduction was present. Partial heart block and complete localization of the infarct and the extension into the septum were confirmed at post-mortem examination.

The second patient was a man aged 52 years who had possible mild angina pectoris for several months before the acute infarction occurred. Large, late R waves were again recorded from the right precordium when the right branch of the His bundle was blocked and large Q or QS waves appeared in all of the precordial leads. When left branch block developed, tracings taken from the left precordium resembled those recorded over the right precordium during the period of right branch block; prominent waves appeared in Lead I. The precordial electrocardiogram during normal conduction suggested that the infarct was anteroseptal in location. The clinical course was complicated by paroxysmal auricular fibrillation but a satisfactory recovery occurred.

In anterior myocardial infarction complicated by intraventricular block, the ventricular complexes recorded from the precordial area overlying the ventricle which is activated late are very similar in form in both right and left bundle branch block in those cases in which the myocardial lesion also involves the interventricular septum.

#### 65. ELECTROCARDIOGRAPHIC CHANGES AFTER ELECTRIC SHOCK TREATMENTS

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In persons with suspected heart disease, but normal electrocardiograms, it has been possible to produce abnormalities in the electrocardiograms by partial oxygen deprivation (Levy) or by measured exercise (Masters). The cases presented here were patients with definite electrocardiographic abnormalities who were subjected to the extreme anoxemia and violent exercise

produced by psychiatric electric shock treatments. To the surprise of the writer, the electrocardiograms assumed a more normal pattern for a few minutes after the treatment.

Case 1 was a man 47 years of age who had suffered a coronary occlusion four years previously. Before treatment the electrocardiogram showed low to flat T waves in Lead I and slightly inverted T waves in Lead II. Immediately after treatment, the inverted T waves became flat.

Case 2 was a 59-year-old woman who had hypertension and a partial hemiplegia. An electrocardiogram showed sharply inverted T waves in Leads I and CF IV and V. Immediately after electric shock treatment the previously inverted T waves had become flat to upright. One minute after treatment they were slightly inverted, two minutes after treatment they were more inverted, and three minutes after treatment they were just about the same as before treatment.

Case 3 was a 41-year-old man who had had a coronary occlusion two months before electric shock treatment. The electrocardiogram showed sharply inverted T waves and deep Q waves in Leads II and III. Immediately after treatment there was a partial cardiac arrest, the rate falling to 20 per minute. The T waves remained inverted. However, the QRS complexes became upright to diphasic.

Case 4 was a 73-year-old woman whose physical examination was negative and who gave no history of cardiac disease. Her electrocardiogram before treatment showed inversion of the T waves in Lead CF IV. Immediately after treatment the T waves became upright. They remained upright for three minutes, then became flat and remained flat for another ten minutes, and at the end of twenty minutes they had become inverted and had resumed their pre-treatment configuration.

Case 5 was a 40-year-old woman with no history of cardiac disease. The T waves were found to be low to flat in Lead I and flat in Lead CF IV. Immediately after treatment the T waves in Lead I had become upright. They remained upright for three minutes and then resumed their previous flat condition.

The small number of cases presented do not warrant any conclusions. It may be said that the increase in amplitude or return to normal of the T waves are the physiologic response to exercise, such as one sees in normal athletic hearts. However, this is not the response in the Masters test, and the foregoing may be considered an extreme exaggeration of this test.

As compared with the tests of Masters and Levy, these results seem paradoxical.

## 66. THE EFFECT OF A SINGLE INSPIRATION IN LEAD III AND LEAD CF IV OF THE HUMAN ELECTROCARDIOGRAM

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The effect on the electrocardiogram of a single deep inspiration during Leads III and CF IV was studied in order to (1) ascertain the frequency and type of respiratory changes produced; (2) determine if any correlation exists between these changes and the presence or absence of heart disease; and (3) determine the influence of digitalis on these respiratory variations. Two thousand three hundred seven consecutive tracings were obtained for this study.

An initial increase in rate followed by slowing of various degrees frequently occurred, but in very few instances was it possible to demonstrate changes in the auriculoventricular conduction with the changes in rate. Digitalis was associated with the most striking changes in rhythm including usually a shift of the pacemaker from the sinoauricular node toward the auriculoventricular node, but the depression of the pacemaker also occurred in the absence of digitalis.

Numerous changes including variations in contour of P waves, QRS complexes, and T waves in Lead III were almost as frequent in normal as in abnormal records. The QRS complexes usually returned to their original configuration very shortly after the T waves assumed their previous form.

In Lead III of normal records there was a decided tendency for inspiration to result in increasing positivity of all deflections, but in Lead CF IV there was a tendency for inspiration to cause increasing negativity of all deflections. In abnormal electrocardiograms, except in those of patients with acute myocardial infarction or in those of subjects under digitalis therapy, inspiration tended to increase the positivity of all deflections in Lead III. In the patients who received digitalis, or who had acute myocardial infarction, inspiration tended to increase the negativity of the T waves in Lead III with an opposite effect on the other deflections. The frequency of T-wave changes in Lead CF IV with inspiration detracts from the significance of the T wave in the fourth lead in the interpretation of borderline records.

Respiratory effects on the electrocardiogram include changes in axis, in T waves, and in cardiac mechanism. It has been suggested that the axis changes are probably the result of extracardiac factors, that T-wave changes are probably the result of intracardiac factors, and that variations in cardiac mechanism are the result of nervous reflexes, chiefly vagal.

## 67. AN UNUSUAL VAGOVAGAL REFLEX TYPE OF ADAMS-STOKES SYNDROME

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(INTRODUCED BY MAURICE HARDGROVE, M.D.)

A patient with a vagovagal type of reflex Adams-Stokes syndrome associated with an anterior traction diverticulum of the esophagus was studied. Spontaneous attacks occurred when cardiospasm caused irritation of the diverticulum through dilatation of the esophagus, and the vagal reflex sensitivity was increased by digitalization. Minor clinical attacks and electrocardiographic evidence of cardiac slowing or arrest occurred in the digitalized patient on distention of a balloon inserted in the esophagus to the level of the diverticulum, but not above or below that level. Right carotid sinus stimulation caused the same phenomenon. Atropine abolished these findings through interruption of the vagal reflex. Withdrawal of digitalis resulted in decreased sensitivity of the carotid sinus and vagovagal reflex irritability to a point where the clinical and electrocardiographic findings were not obtained. Intravenous administration of 0.8 mg. of digitoxin caused re-establishment of the carotid sinus and vagovagal reflex irritability within four hours.

This is the second case, known to us, of Adams-Stokes syndrome due to vagovagal reflex associated with an esophageal diverticulum and the first in which the summation role of digitalis in producing the syndrome has been reported. This summation role of digitalis in reflex types of cardioinhibitory Adams-Stokes syndrome seems worthy of note.



## 68. THE VARIABILITY OF THE FASTING NOCTURNAL GASTRIC SECRETION IN NORMAL INDIVIDUALS AND IN PATIENTS WITH DUODENAL ULCER

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The fasting nocturnal gastric secretion was measured in thirty-three normal individuals and in thirty-two patients with active duodenal ulcer. The average twelve-hour volume, concentration of hydrochloric acid, and total output of acid were significantly higher in patients with duodenal ulcer than in normal individuals. In the normal group, the volume averaged 581 ml; the free acidity, 29 clinical units; and the total output of acid, 661 milligrams. In the duodenal ulcer series, the volume averaged 1,004 ml.; the free acidity, 61 clinical units; and the total output of acid, 2,242 milligrams. Similarly, the average hourly secretion was persistently higher in patients with duodenal ulcer than in normal individuals. Wide individual variations were observed in both groups.

In addition, considerable spontaneous fluctuation in the hourly and total night secretion was noted in the same individual. Thus, in the normal subjects, the average percentage variations in the volume, free acidity, and total output in the same individual were 24, 44, and 47 per cent, respectively. In patients with duodenal ulcer the average spontaneous variation was: volume, 17 per cent; free acidity, 24 per cent; and total hydrochloric acid output, 23 per cent. The volumes in both groups were significantly higher during the first six hours of the night as compared with the last six hours. There was no important difference in the average concentration of free hydrochloric acid during the various periods of the night in the normal subjects, whereas in the duodenal ulcer series, the output of acid was significantly lower during the last three hours of the night than at any other time.

These variations occurring spontaneously in the same individuals must be taken into account in the proper evaluation of procedures utilized for the purpose of reducing gastric secretion.

## 69. ATROPHIC GASTRITIS AND MALNUTRITION

LEONIDAS H. BERRY, M.D., CHICAGO, ILL.

Most of the conceptions and investigations into the causes of gastritis in the past have been concerned with extrinsic factors and local irritation. It is probably true that next to the skin the organ which is subjected to the most rigid abuse from man's external environment is the stomach. Today investigators show a definite trend of interest in the role of intrinsic factors in gastric disease. Psychosomatic factors are being emphasized.

Our studies of patients with chronic atrophic gastritis show the etiologic importance of malnutrition and indicate that most of the chronic atrophic gastritis coming under our observation is probably primary rather than inflammatory. Malnutrition not due to disease, most commonly but not invariably, follows an economic pattern. This fact was very striking in our studies.

The analysis of data on 706 dyspeptic patients gastroscoped 1,000 times during four years immediately preceding World War II showed some striking differences when compared with 297 patients with dyspepsia gastroscoped 364 times during the four war years. In the "prewar" group (1937 to 1942) 53.6

per cent of the total chronic gastritis seen was the atrophic type. The remaining 46 per cent was about equally distributed between the chronic superficial and chronic hypertrophic types. The "wartime" group (1942 to 1946) showed a considerable decline in chronic atrophic gastritis (from 53.6 per cent to 18.5 per cent) and a corresponding rise in the superficial and hypertrophic types. Thus chronic "atrophic gastritis" was three times higher in the "prewar" group. What is the significance of this variation in distribution? Rather extensive laboratory studies were done. A large percentage of the "prewar" group had dietary questionnaire and food habit studies. Ninety per cent of the "prewar" group were clinic patients, unemployed from two to four and up to eight years prior to examination. Nutrition diaries clearly showed that most of these patients existed on diets inadequate in quantity and quality of protective foods to maintain normal nutrition. There were evidences of low or borderline plasma ascorbic acid levels, red blood cell counts, hemoglobin values, spongy gums, underweight, and other evidences of subclinical states of under-nutrition. Symptomatically those patients with so-called atrophic gastritis were fatigued easily and had attacks of weakness in addition to abdominal distress.

As employment improved the ratio of patients on relief to those gainfully employed was reversed. Therapeutic studies (reported in another communication) making use of liver extract, vitamin B complex, and improved diets showed striking symptomatic improvement and convincing disappearance of atrophy. These facts contribute further evidence to the etiologic relationship of malnutrition to so-called chronic "atrophic gastritis."

## 70. THE EFFECT OF CHOLINE, METHIONINE, AND LOW FAT DIET ON THE LIFE EXPECTANCY OF PATIENTS WITH CIRRHOSIS OF THE LIVER

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(INTRODUCED BY CARL HARFORD, M.D.)

In a previous communication the following regimen for the treatment of cirrhosis was described:

1. Diet
 

Protein	90-100 grams
Fat (chiefly vegetable)	30 grams
Carbohydrate	Sufficient for patient's caloric needs
2. Drugs
 

Choline hydrochloride	1-8 grams
Methionine (1 quart skimmed milk)	0.9 grams
3. Vitamins
 

A	10,000 U. S. P. units
D	800 U. S. P. units
4. Abstinence from alcohol

This regimen was based on two assumptions: (1) that fatty changes in the liver ordinarily precede the more characteristic anatomic changes of cirrhosis; (2) that fatty changes in the liver are often dietary in origin, especially in the alcoholic patient, and are comparable to the changes observed in experimental animals placed on deficient diets. The present report summarizes the clinical experiences of the past six years, during which time some 224 patients with cirrhosis of the liver have been studied. The fate of these patients is compared with that of a similar group of 311 patients treated in the same hospital under similar circumstances prior to the use of the present therapeutic regimen.

The fate of the two groups of patients, as of March 1, 1947, is as follows:

	EXPERIMENTAL		CONTROL	
	NUMBER	PER CENT	NUMBER	PER CENT
Dead	168	77.1	265	85.2
Living	44	20.2	7	2.3
Fate unknown	12	2.7	39	12.5
Total	224	100.0	311	100.0

Comparison of the survivors in the two groups permits no conclusion regarding the merits of the therapeutic regimen since the period of observation of the experimental group has been too brief. The period of survival thus far from the onset of initial symptoms varies from 13.0 to 63.2 months for the experimental series as compared with 71.5 to 167.5 months for the control series. The average period of survival is 33.0 months and 112.2 months, respectively.

It is not possible to utilize all the patients known to be dead in the two series since some were thought to have died from causes not directly related to their liver disease. Adequate data regarding the onset of symptoms were not available in other instances. The experimental series is thus reduced to 138 patients and the control group to 196 cases.

If one plots the percentage of patients surviving at any given time after the onset of the initial symptoms, the curves for the experimental and control groups superimpose one upon the other. Similar curves comparing the survival rate after the onset of ascites, jaundice, or hemorrhage also reveal no difference between the experimental and control groups.

It is concluded, therefore, that the therapeutic regimen as carried out was without effect upon the life expectancy of patients with cirrhosis.

## 71. HEPATIC FUNCTION IN INFECTIOUS MONONUCLEOSIS

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JACK E. CLIFFORD, M.D., MADISON, WIS.

(INTRODUCED BY OVID O. MEYER, M.D.)

Liver function tests were performed one or more times in forty-eight consecutive cases during the course of infectious mononucleosis. The presence of infectious mononucleosis was established with reasonable certainty according to existing criteria.

The following tests were made one or more times on each patient with the results indicated: icterus index in 43 patients, abnormal values obtained in 16; urine urobilinogen in 33, elevated values in 20; cephalin cholesterol flocculation test in 47, value of 3 plus or more in 37; thymol turbidity in 41, value of 4 units or more in 16; prothrombin time in 41, levels below 75 per cent in 5; 2 mg. per kilogram twenty-minute bromsulfalein test in 21, 10 per cent or more retention in 1. Certain tests were performed three or more times in several patients. Of 24 patients who had the cephalin cholesterol flocculation test three or more times, 23 (96 per cent) attained a value of 3 plus or more at least once. Eleven of twenty patients who had thymol turbidity tests three or more times attained a level of 4 units or more at least once. For the patients who had a series of observations, the average period from onset of disease to last abnormal value for Hanger's test was 34.7 days, and for thymol turbidity test, 38.9 days. Persistence of symptoms was noted for a variable period after the liver function tests performed were normal in seven of fifteen patients with adequate follow-up. The tests to date have not revealed a significant correlation between the heterophile antibody reaction and Hanger or thymol turbidity test.

## 72. EXPERIENCES WITH NEEDLE BIOPSY OF THE LIVER IN PATIENTS WITH MALIGNANT HEPATIC NEOPLASM

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CINCINNATI, OHIO

Needle biopsy of the liver was performed with the Vim-Silverman needle in forty-seven patients with proved malignant neoplasm of the liver. The transpleural approach was used in thirty-six of the patients and the abdominal approach in eleven. The tumor was primary in ten and secondary in thirty-seven. Neoplasm was demonstrated in the tissue obtained from thirty-nine of the group. The tumor was missed in eight biopsies, in three of which the specimen obtained was inadequate for microscopic examination.

The liver was palpated as nodular or irregular in twenty-nine of the patients and as smooth in seventeen. In one patient it was not palpable. The clinical diagnosis of malignant neoplasm of the liver was confirmed by means of the biopsy in thirty cases. In nine patients, three with primary hepatic tumor, carcinoma was not suspected prior to the biopsy. In sixteen of the twenty-nine patients with nodular liver, and in twelve of the twenty with smooth liver, the biopsy was the first means by which the presence of carcinoma was definitely established.

Liver biopsy proved particularly valuable in patients in whom the presence of carcinoma was not previously established and in whom "liver function" studies, especially the cephalin cholesterol flocculation test, suggested the presence of hepatitis.

Needle biopsy of the liver is a surprisingly valuable adjunct in the diagnosis of primary and secondary neoplasm of the liver and not infrequently may be the earliest or the sole means of establishing the diagnosis.

## 73. INFECTIOUS AND TOXIC HEPATITIS: PATHOLOGIC AND CLINICAL MANIFESTATIONS

HANS POPPER, M.D., FREDERICK STEIGMANN, M.D., MURRAY FRANKLIN, M.D. (BY INVITATION), AND LEO F. NARUT, M.D. (BY INVITATION), CHICAGO, ILL.

Studies in the Army Institute of Pathology on fatal cases of infectious (viral) hepatitis revealed a characteristic morphologic picture. Investigation of fatal cases of acute primary hepatitis observed in a civilian hospital during eighteen years showed in less than one-half a morphologic picture similar to the one seen predominantly in military personnel; these cases were considered to be infectious in origin. Since in many of the other cases a hepatotoxic etiologic factor was present, these were tentatively designated as toxic hepatitis. The main morphologic differences were slower cell death, slighter mesenchymal involvement, outspoken zonal distribution, and occasionally predominant fatty changes in the toxic cases. Applying similar differentiating points to biopsy specimens of thirty-seven cases of acute primary nonfatal hepatitis, usually a differentiation between infectious and toxic hepatitis was possible. Subsequently a correlation with the apparent etiologic factors was found. More than one-half of the studied cases revealed a morphologic picture designated as toxic.

Based on this differentiation, we attempted to divide the cases of acute primary hepatitis by clinical and laboratory methods. For instance, usually patients with toxic hepatitis are older and have a shorter prodromal period with

more intense gastrointestinal symptoms (vomiting and diarrhea) which continue even while the patient is in the hospital in contrast to infectious hepatitis in which the gastrointestinal symptoms decrease when jaundice appears. The patients with toxic hepatitis appears sicker, more feverish and dehydrated, are more often deeply icteric, and have tachycardia. Pruritis and dermatitis may be present. The spleen is more often felt. There is no clear-cut differentiation in the laboratory findings. In general, however, cephalin flocculation and thymol turbidity are more often negative and alkaline phosphatase and total cholesterol more often elevated in the toxic cases. In toxic hepatitis, the non-protein nitrogen is often elevated and in the fatal cases icteric nephrosis is common, some of the patients dying in uremia. The dehydration in toxic hepatitis may be held responsible for the renal involvement just as dehydration has been considered an important pathogenetic factor in lower nephron nephrosis.

Definite establishment of the etiologic factors in acute hepatitis by virus studies might justify the thus far tentative designation of infectious and toxic hepatitis. The present findings suggest two different entities and that not every case of acute primary hepatitis in civilians is of infectious origin.

#### 74. METHIONINE IN THE TREATMENT OF CHRONIC AND ACUTE HEPATITIS

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Encouraged by results of treatment of experimental nutritional cirrhosis by lipotropic substances, methionine, choline, or choline plus cystine were used as supplement in patients with liver cirrhosis. While the reports on such regimes are few and on small series of cases, most of them are rather optimistic.

In a comparative study of different lipotropic substances during the past two years, a group of ten patients with hepatic cirrhosis received, in addition to a high protein, high carbohydrate, high vitamin, and low fat diet, daily 5 Gm. of methionine orally. Seven of the patients had ascites; all with the exception of three were jaundiced; their symptoms varied from ten days to five years. They were treated in the hospital from ten days to five months. Three patients died during the first stay in the hospital and seven recovered and were discharged. Of these, two returned six and twelve months later, respectively, and died during the second admission. Of the remaining five, two are still ailing, while three have returned to their previous occupation, at which they have remained so far nine to twelve months. The jaundice disappeared in the recovered cases; the ascites decreased in two, and in three paracentesis is still occasionally necessary. The liver function improved in the surviving cases, especially the albumin/globulin ratio and the cephalin flocculation. Some improvement of liver function occurred even in two patients who later died.

Comparison of the results of this group with another without lipotropic supplement reveals a definite improvement both as to mortality and recovery. It should be considered that the variations in the therapeutic effects are not only due to different clinical stages, but also possibly to different types of cirrhosis.

The evaluation of therapeutic results in acute hepatitis is difficult because of the erratic course of the disease. It appeared therefore more revealing to study the influence of methionine upon the nitrogen balance in patients with acute infectious hepatitis. In four such patients no protein-sparing effect was found in experiments composed of several five-day periods. These results

speaking against the existence of a methionine deficiency in acute infectious hepatitis due to diversion of methionine from protein formation to lipotropic or other liver-protecting activity.

## 75. PROGNOSTIC VALUE OF BIOPSY IN CIRRHOSIS OF THE LIVER

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(INTRODUCED BY THOMAS FINDLEY, M.D.)

Liver biopsies were accomplished in twenty-one patients with cirrhosis either by needle or surgical approach. Follow-up data were available in sixteen of these. In this paper an attempt has been made to determine the prognostic value of changes in the liver by correlating the degree of fatty change, fibrosis, necrosis, and inflammation in the liver sections with the response to therapy. The relation of ascites and the presence or absence of central veins was also noted. Treatment consisted of high carbohydrate (500 Gm.), high protein (200 Gm.), variable fat diet with supplements of brewer's yeast, vitamins, liver extract, and, in some instances, methionine or choline.

Of the group, eleven were considered to be decompensated and of these, regardless of the degree of fibrosis, necrosis, and inflammation, only those with rather severe fatty change showed significant improvement under treatment. In the compensated group microscopic changes were relatively minor and the clinical course was correspondingly good. In general, those patients with necrosis and inflammation showed better clinical and microscopic improvement than those with only fibrosis. The two patients with severe necrosis and inflammation without fatty change both died during the acute episode. Four other patients with similar changes plus considerable fatty infiltration have returned to useful life. In general, the patients with ascites showed relatively fewer central veins than those without ascites.

## 76. AN UNUSUAL CASE OF ESSENTIAL NORMAL CHOLESTEREMIC XANTHOMATOSIS

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Extensive biochemical, roentgenographic, electroencephalographic, and histologic studies have been made in the case of a 38-year-old white man with cutaneous xanthomatosis and grand mal, uncinate, and psychomotor seizures.

The initial manifestations of the disease appeared two and one-half years before. There was no history of seizures prior to the development of xanthomatosis and no family history of seizures.

Electroencephalograms showed diffuse cerebral disorder with some left-sided localization, and marked abnormalities were elicited following left carotid pressure which produced temporary asystole and faintness.

Total cholesterol, 196 mg.; free, 48 mg., cholesterol esters, 148 mg. per 100 ml. of blood.

Roentgenograms of the skull were normal. The long bones showed medullary thickening in the lower portion of the shafts of the femora and humeri but no punched-out areas. Biopsy of the femur showed xanthomatous involvement.

The most unusual feature of the case was the finding of scattered petechiae and small raised mahogany lesions over the limbs and trunk which tended to

appear in crops and fade in a few weeks. In addition, he showed classical chamois-colored lesions involving the lids and the entire scalp. Biopsy of one of the petechiae showed infiltrations of reticuloendothelial cells and eosinophiles around small blood vessels. Such lesions are extremely rare in adults with xanthomatosis of this type.

The patient suffered a pathologic fracture near the biopsy site after release from the hospital. His seizures have been satisfactorily controlled with dilantin sodium.

This case appears to represent essential xanthomatosis of the normal cholesteremic type (eosinophilic granuloma, eosinophilic xanthomatous granuloma) involving skin, bone, and dura, with eosinophilic granulomas involving the small blood vessels of the skin.

## 77. "COMBINED ANTACID" THERAPY IN PEPTIC ULCER

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The large number of antacids available for the treatment of peptic ulcer indicates that no one substance is of value in all cases. The disadvantages of some of the currently used antacids are acid rebound, alkalosis, too-transient neutralization, interference with normal gastric function, and unpalatability. Hence, the search for better antacids continues. Recently we have studied the antacid effect of a substance which has been prepared with the idea of combining the properties of currently used antacids but eliminating some of the untoward effects. This substance is a mixture of aluminum hydroxide, magnesium trisilicate, and purified gastric mucin.

Twenty-five patients with symptomatology and x-ray evidence of ulcer (twenty duodenal and five gastric) were chosen for this study. The fasting stomach was aspirated at fifteen-minute intervals for one hour before 0.5 mg. of histamine phosphate was injected subcutaneously. Following this, five further aspirations were done. On successive days, two tablets of the new preparation, of amphogel, or two Sippy tablets were chewed and swallowed following the fourth fasting aspiration and prior to the histamine injection. The free and total acidity was determined in all samples by titration with 0.1 N sodium hydroxide solution, using Töpfer's solution and phenolphthalein as indicators.

In 92 per cent of the cases, a variable antacid effect was obtained with all substances. In 55 per cent, the mucin mixture was more effective; in 27 per cent, less effective. In 18 per cent, they were equally effective. In 70 per cent of the cases, the mucin preparation was observed in the aspirated samples from fifteen to forty-five minutes longer than the other antacids. Recent gastroscopic and x-ray studies by Hardt confirmed this observation by demonstrating that the mucin preparation remained in the stomach longer. It was also noted that the mucin mixture was a more effective antacid when chewed than when allowed to dissolve in the mouth.

The preliminary antacid studies with this preparation which are now being supplemented by clinical observations suggest that this substance may prove a valuable addition to peptic ulcer therapy.

## 78. TRANSITION OF PANCREATIC EDEMA INTO PANCREATIC NECROSIS

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Pancreatic edema or pancreatic necrosis can be produced easily in the dog, but we have not been able, heretofore, to observe edema progress or change into necrosis. This has been achieved now by temporary clamping of the upper pancreatic artery in the presence of edema of the pancreas. It is felt that spasm of arteries may play a role in the development of pancreatitis.

## 79. BIOCHEMICAL FINDINGS IN ACETYL SALICYLATE THERAPY OF ACUTE RHEUMATIC FEVER

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Eighty patients with acute rheumatic fever were treated with high doses of aspirin combined in tablet form with colloidal aluminum hydroxide (Alasil, Wander). Preliminary tolerance studies had shown that the plasma salicylate levels obtained after single doses of the aspirin product were of the same order as those obtained with ordinary aspirin. But the gastrointestinal disturbances obtained in 13 per cent of the patients on high doses of ordinary aspirin did not occur with the aspirin containing aluminum hydroxide.

From 94 to 144 grains of aspirin daily (about 120 mg. per kilogram) usually produced plasma salicylate levels around 30 mg. per 100 milliliter. At this level, symptoms of salicylism often occurred with an accompanying irresponsibility and negativism. Many patients refused further to cooperate with the treatment and these had to have the dosage lowered. Thus in only forty-one patients was an average level of more than 25 mg. per 100 ml. maintained. The patients were treated until subsidence of clinical symptoms.

There was no great difference in the percentage of patients whose sedimentation rate subsided to normal, whether average salicylate level was above or below 25 mg. per 100 milliliter.

The plasma prothrombin levels were not much altered. They were reduced below 75 per cent of normal in only 33 per cent of the cases. In only one patient was the level low enough to produce bleeding, which was relieved by one single injection of vitamin K. There was no greater incidence of reduced prothrombin concentrations in the patients with high plasma salicylate concentration than in those with lower levels.

In almost all patients, a moderate reduction in serum bicarbonate occurred, usually associated with increased chloride concentrations and with slightly lowered sodium concentrations. The serum pH was usually normal. This might have been due to primary alkalosis produced by hyperventilation, but there was no marked hyperpnea, the urine was never alkaline, and clinical improvement occurred with bicarbonate. These findings along with those obtained with salicylate and chloride clearance studies pointed to the idea that salicylate and chloride compete for sodium in renal excretion and that when sodium is limited both are retained to produce a primary fixed acid acidosis.



# 80. TREATMENT OF SUBACUTE BACTERIAL ENDOCARDITIS WITH PENICILLIN IN OIL AND BEESWAX

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Eight patients with all the recognized clinical criteria for subacute bacterial endocarditis were treated with daily single injections of 2 ml. of hard Romansky formula penicillin (crystalline potassium penicillin in peanut oil and beeswax\*) containing 600,000 units. Six of the patients had one or more positive blood cultures from which pure strains of *Streptococcus viridans* were isolated. No bacteria could be isolated from the remaining two patients. The treatment was continued for forty-two days, the daily injections being given in alternate buttocks. All of these patients had histories and findings of an underlying rheumatic heart disease.

In these eight patients, the treatment was successful. Remission of fever and clinical symptoms occurred early in the treatment, in some cases during the first week. Cultures became negative in all cases, in some cases before the end of the first week, in no cases later than three weeks. Usually there was gain in weight and strength and ameliorization of anemia. One patient with right hemiplegia on admission from cerebral embolism had severe malnutrition and bedsores during the early part of the treatment, even though the cultures became negative. When her diet was supplemented with intravenous amino acids, glucose, and vitamins B and C, she improved rapidly. There were no urticarial reactions to the many injections. In one obese patient, the induration at the sites of injections required heat to alleviate pain. In no other cases were there any significant local or general reactions. The penicillin sensitivity of the isolated bacteria ranged from 0.008 to 0.3 units per milliliter. Plasma penicillin levels ranged from 1 to 4 units at two hours to values of 0.03 to 0.125 at twenty-four hours. Only one zero level was obtained at twenty-four hours.

A ninth patient with subacute bacterial endocarditis from a focus in the bladder and posterior urethra had *Str. viridans* in the blood with a penicillin sensitivity of 4.15 units per milliliter. He was treated with daily injections of 3 ml. of Romansky penicillin (900,000 units) for thirty-four days without success. After a change to 500,000 units of aqueous penicillin every three hours intramuscularly, the patient still had a positive culture on the fourth day of the new treatment.

The use of Romansky formula penicillin for the treatment of subacute bacterial endocarditis is thus a convenient and successful method if the organism is not too penicillin-resistant.

# 81. TREATMENT OF LOBAR PNEUMONIA WITH PENICILLIN IN OIL AND BEESWAX

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(By Invitation), AND WILLIAM S. HOFFMAN, M.D., CHICAGO, ILL.

One hundred four consecutive patients entering the Cook County Hospital with lobar pneumonia were treated with daily injections of hard Romansky formula penicillin (crystalline potassium penicillin G in peanut oil and beeswax\*). The daily dosage was 1 ml. containing 300,000 units. Seventy patients had typable pneumococci in the sputum; of the remaining thirty-four, some almost certainly had primary virus infections. Sixty-two per cent of all the

\*Commercial Solvents Corp., New York, N. Y.

patients showed a satisfactory remission of fever and symptoms within seventy-two hours. Many of these showed this response after one injection. The percentage of good results was somewhat higher in the group of proved pneumococcal origin. There were four deaths, these occurring in patients with serious complications and of the older age groups. Many of the patients who responded slowly had handicapping complications, such as delirium tremens, chronic heart disease, chronic lung infections, and cirrhosis. There was only one urticarial reaction to the penicillin and no local abscesses.

Plasma penicillin concentrations obtained in twenty-five hospital control patients who were administered 300,000 units of the penicillin in oil and beeswax averaged 0.43 unit per milliliter at two hours, 0.11 at six hours, and 0.09 at twenty-four hours. There were three zero levels at twenty-four hours. In the 104 therapeutic cases, 122 determinations at twenty-four hours showed seven zero levels, the bulk of the values lying between 0.03 and 0.125 unit per ml., with an average of 0.10. At two hours the average level was 0.85 unit per milliliter. Since almost all pneumococci are sensitive to concentrations of 0.03 unit per milliliter or less, the levels obtained over the twenty-four hours with the single daily injections should have been therapeutically satisfactory.

The satisfactory results in over 50 per cent of the bacteriologically unproved cases of pneumonia suggests that penicillin therapy is indicated in all cases of pneumonia. However, if no satisfactory results are obtained in forty-eight hours, the case should be clinically re-evaluated.

## 82. LABORATORY AND CLINICAL OBSERVATIONS ON THE USE OF A NEW SULFONAMIDE—3,4-DIMETHYL-5-SULFANILAMIDO- ISOXAZOLE (NU 445)

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In vitro comparisons of the bacteriostatic activity of sulfadiazine and NU 445 were carried out with synthetic media to eliminate the polypeptide inhibitory factor. It was found that the two sulfa drugs studied were roughly comparable in action against *Escherichia coli*, gamma streptococcus of fecal origin, and hemolytic streptococcus. The acetylated forms of the drugs inhibited the growth of *Esch. coli* and gamma streptococcus but required slightly higher concentrations than the free drug. Folic acid was found greatly to inhibit the activity of both sulfadiazine and NU 445 against the gamma streptococcus but not against *Esch. coli*.

In twenty-four patients receiving NU 445 orally and parenterally in doses of 4 to 6 Gm. daily, the average blood level (fifty-three determinations) was 9.10 mg. per cent total and 6.36 mg. per cent free drug. About 30 per cent of the drug was therefore present in the N, conjugated form (acetylated), a figure considerably higher than for acetylated sulfadiazine or sulfamerazine as determined in this laboratory. In two instances in which NU 445 levels were determined on blood and spinal fluid from the same patient obtained the same day the spinal fluid levels were approximately one-half those of the blood levels.

Slightly more than one-half of the drug administered was recovered in the urine, about 35 per cent being present in the acetylated form. These figures closely approximate those obtained with sulfadiazine.

NU 445 was used clinically in 24 cases, including 6 patients with meningitis (meningococcal, 3; pneumococcal, 2; undetermined etiology, 1), 6 with urinary tract infections, 5 with respiratory infections, 2 patients with erysipelas, 1 with

scarlet fever, and 4 with postoperative infections. In 14 the drug was used alone and in 10 it was used in combination with penicillin and/or streptomycin. In no case was a patient's death thought to be due to failure of the drug to control the infection. Both patients with meningococcus meningitis treated with NU 445 alone recovered.

Reactions occurred in four cases, consisting of a generalized dermatitis in one, crystalluria and hematuria in one, and nausea and vomiting in two others. No local reaction resulted from repeated intramuscular injection of the drug (lithium salt) in 10 per cent solution.

### 83. CLINICAL STUDIES OF INSOLUBLE KETONES AS INTESTINAL ANTISEPTICS

#### PRELIMINARY REPORT

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Since the discovery of the properties of sulfaguanidine by Marshall, Bratton, White, and Litchfield in 1940, it has been shown that many other sulfonamide groups had similar intestinal antiseptic properties. The clinical advantage claimed for the use of compounds that are absorbed slightly or not at all from the intestine is the obviation of side effects.

Recently a new group of compounds was discovered that are absorbed very slowly from the intestine and yet demonstrate antibacterial activity. The group is derived from p-aminobenzene sulfonic acid (a series of insoluble ketones). Ketones Nu201 and Nu404 were studied. The toxicity values are expressed as a single oral dose of 20 Gm. per kilogram tolerated by white mice for ketone Nu201 and 15 Gm. per kilogram for ketone Nu404. We have administered the new agents to patients orally in doses of one tablet three times daily before meals. Ketone Nu201 was administered over a period from seven to twelve days and Nu404 from eleven to forty-nine days. The blood levels for Nu201 ranged from .08 to 1.2 mg. per milliliter and those for Nu404 from .09 to .8 mg. per milliliter.

In our experience Nu201 is apparently toxic to some patients.

Bacteriologic studies in the stools showed a reduction in the intestinal flora in the staphylococci and streptococci groups. The studies on the *Escherichia coli* group were inconclusive. Clinically, the patients experience a reduction in the number of stools per day, disappearance of abdominal cramping, and a decrease in the amount of blood in the stools. In general, our preliminary experience with Nu404 is more favorable. More work is needed to confirm and to extend our present findings.

### 84. THE COMPARATIVE EFFECT OF VARIOUS ALKALIS AND UREA UPON THE BLOOD CONCENTRATION, EXCRETION, AND CON- JUGATION OF SULFONAMIDES WITH GLUCURONIC AND ACETIC ACID

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The most valuable sulfonamide adjuvant would be one which enhances glucuronic acid conjugation, depresses acetylation, and prevents crystalluria. Since sulfonamides conjugated with glucuronic acid are less likely to result in

uroliths and are more antibacterial than acetylated forms according to the literature, it seemed worth while to study the effect of various adjuvants on the conjugation of sulfa drugs with glucuronic and acetic acid.

Sulfadiazine was given orally to one patient and sulfamerazine to the other on alternate three-day periods for two months with various adjuvants. Sodium citrate and sodium lactate dosages were equivalent to 24 Gm. of sodium bicarbonate daily and the dosage of urea was 60 Gm. per day. Glucuronic acid and the sulfa fractions were determined in blood and urine.

Total sulfonamide blood concentrations were slightly reduced by the alkali adjuvants but not by urea. No significant effect of adjuvants on the percentage acetylated was demonstrated.

In most instances blood glucuronic acid was increased by sodium citrate and sodium lactate adjuvants but not by sodium bicarbonate, nor the sulfonamide alone. Urea depressed the blood concentration of glucuronic acid.

Urinary glucuronic acid was increased by both sulfadiazine and sulfamerazine either with or without adjuvants.

In general there were greater increases of urinary glucuronic acid after alkali adjuvants were administered than when urea or no adjuvant was given. These increases above controls, which were taken on days that adjuvants only were given, were calculated to represent equimolar conjugation with sulfonamide.

The percentage of urinary acetylated sulfonamide was less when the alkali adjuvants were given than when urea or no adjuvant was given.

Urea had no effect on blood concentration, excretion, or conjugation. Most of the ingested urea was recovered in the urine. Blood and urine urea nitrogen had returned almost to control levels twenty-four hours after stopping the drug.

The fact that sodium citrate and sodium lactate increase conjugation of sulfadiazine and sulfamerazine with glucuronic acid and decrease conjugation with acetic acid is an additional indication for the use of adjuvant alkali therapy with these drugs.

## 85. CRYSTALLURIA FOLLOWING SULFONAMIDE MIXTURES AND SULFONAMIDES PLUS ALKALI: A COMPARATIVE STUDY OF 400 CASES

LEOPOLD J. SNYDER, M.D., ELOISE, MICH.

(INTRODUCED BY CHARLEY J. SMYTH, M.D.)

The use of sulfonamides is attended by the danger of renal complications. Lehr (1945) and Flippen (1946) showed that using a mixture of two sulfonamides markedly reduced crystalluria. This occurs because sulfonamide solutions are mutually soluble. Flippen demonstrated that using single sulfonamides, 12 Gm. of sodium bicarbonate are necessary to keep crystalluria incidence equal to that of sulfonamide mixtures.

This study compares the incidence of crystalluria in 200 patients using a mixture of sulfadiazine and sulfamerazine in equal doses to the crystalluria in 200 patients using a sulfonamide with equal doses of sodium bicarbonate. Group I was given a daily total sulfonamide mixture dose of 4 to 6 grams. Group II received 4 to 6 Gm. of sulfadiazine or, in a few cases, sulfathiazole with equal doses of sodium bicarbonate. Group I averaged 59 years in age and

Group II, 52 years in age. Urinalyses were done every other day. No case was included unless the patient had received the drug for at least sixty hours or had developed crystalluria before that time.

In Group I only 20 per cent of the patients developed crystalluria. Those in Group II showed, however, 42 per cent crystalluria. It is further of significance that in Group I, of seventy-three patients over 60 years old, thirteen (22 per cent) developed crystalluria, comparing favorably with the over-all group. In Group II, however, of sixty-four patients over 60 years, thirty-three (52 per cent) developed crystalluria.

It is obvious that the large amount of sodium bicarbonate recommended by Flippen might well be deleterious in many cases, especially in an old age group or in patients with cardiac or chronic renal disease. Although the 20 per cent crystalluria in Group I of this study does not approach the 6 per cent reported by this observer, it is significantly lower than the 42 per cent found in Group II.

It is concluded that the use of a mixture of sulfadiazine and sulfamerazine is superior to the use of a single sulfonamide plus adjuvant sodium bicarbonate because (1) crystalluria is less in all age groups using the mixture and (2) use of the mixture obviates the necessity of using large doses of sodium bicarbonate in cases in which sodium retention is likely.

## 86. RECTAL ABSORPTION OF PENICILLIN

EMANUEL E. MANDEL, M.D., CHICAGO, ILL.

(INTRODUCED BY HANS POPPER, M.D.)

Lack of conclusive information on quantitative penicillin absorption from the rectum and contradictory statements as to its efficacy have prompted investigation of this subject.

Retention enemas containing 0.1 to 1 million units of penicillin usually produced insignificant antibiotic activity in both blood and urine.

Suppositories prepared by mixing 0.5 to 1.0 million units of penicillin with cocoa butter resulted in therapeutically useful serum levels lasting several hours. The average peak of 0.5 to 1.0 unit per milliliter, respectively, occurred about fifteen minutes following administration. The total excretion of penicillin averaged 6 per cent of the dose given.

Rectal insufflation of 200,000 units of penicillin through an anoscope was followed by serum concentrations of up to 8 units per milliliter after fifteen minutes and by recovery from the urine of 17 to 50 per cent of the penicillin blown into the rectum. The actual excretion ratio is probably better because diffusion and adherence of some powder to the instrument prevent part of the drug from reaching the mucosa. Comparable penicillin levels in serum and urine were found to result from cocoa butter capsules containing dry penicillin in their centers (16 to 35 per cent urinary excretion). The similar use of rectal gelatin capsules generally produced relatively lower and delayed penicillin activity in blood and urine, the latter ranging between 1 and 22 per cent of the dose administered.

Rectal absorption of penicillin per se is highly efficient and equivalent to upper intestinal absorption. Distribution of penicillin in solution above the lower rectum, intimate contact of the drug with cocoa butter, and slow or incomplete disintegration of gelatin capsules are inhibiting factors. The usual

rapidity of absorption rules out any significant interference by enzymatic action. The clinical application of the rectal route depends upon the selection of the most suitable vehicle.

## 87. STREPTOMYCIN RESISTANCE OF BRUCELLA SUIIS AND EBERTHIELLA TYPHOSA

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(INTRODUCED BY FORD K. HICK, M.D.)

The streptomycin resistance of microorganisms obtained in blood cultures from one patient with undulant fever and from one with typhoid fever was measured and correlated with the effects of streptomycin therapy.

*Br. suis* was obtained from the blood of the patient with undulant fever six times in ten days before streptomycin therapy and three times in twenty-eight days after streptomycin therapy. Two cultures made during streptomycin therapy were sterile. Treatment with 26.25 Gm. of streptomycin resulting in blood levels of 22 and 25 units per milliliter had no effect on the symptoms of the disease, which were unusually severe. No significant change occurred in the susceptibility of the strains during therapy. All were inhibited in vitro by 1 unit of streptomycin per milliliter with the exception of one slightly more resistant strain that was inhibited by 3 units per milliliter.

*E. typhosa* was obtained from the blood of the patient with typhoid fever on the seventh day of the disease and again on the forty-eighth day during a relapse. A total of 37 Gm. of streptomycin was administered for nine days from the tenth to the nineteenth day inclusive without effect upon the symptoms of the disease. No change took place in the susceptibility of the strains during treatment as both were inhibited in vitro by 3 units of the streptomycin per milliliter of media.

The association of increased bacterial resistance in vitro with lack of therapeutic effect in vivo that is so common in streptomycin therapy of urinary tract infections, *Haemophilus influenzae* meningitis, and tuberculosis was not found in these cases. Further growth of the two microorganisms in media containing streptomycin led to the development of substantial increase of resistance. Other mechanisms for the lack of therapeutic effect of streptomycin on infections caused by susceptible strains of brucella and eberthella, such as inhibition by body fluids or inability of the antibiotic to penetrate to the sites of infection, must be postulated.

## 88. THE EXPULSION OF BETA HEMOLYTIC STREPTOCOCCI BY SNEEZING

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CHICAGO, ILL.

Jennison's stroboscopic photographs have revealed the important component of a sneeze to be its highly atomized oral discharge, its nasal discharge being either absent or very poorly atomized. The importance of sneezing in the transmission of respiratory disease will depend largely, then, upon (1) the size and trajectories of droplets expelled from the mouth and (2) the concentration of a given respiratory pathogen in the oral and, to a less extent, in the nasal discharge.

Very few experimental data exist regarding (1) the numbers of human pathogens expelled into the air when carriers sneeze, or (2) the important question of how far the droplets which contain these pathogens are propelled and whether or not they remain suspended in the air for any length of time. Data on these points in reference to beta hemolytic streptococci were obtained by culturing the air of an experimental room in which carriers sneezed. Tiny "droplet nuclei" which remain floating in air for some time were captured by three "bubbler" air samplers set up 1.5, 5.5, and 9.5 feet from the subject, with each sampler's inlet three feet above the floor. Large rapidly falling droplets were caught on blood agar plates on the floor next to each bubbler sampler.

Fifteen of twenty carriers sneezed hemolytic streptococci in rapidly falling droplets 1.5 feet but not as far as 5.5 feet. These fifteen carriers expelled none as "droplet nuclei." Two others discharged small numbers of hemolytic streptococci as "droplet nuclei" but none in large droplets, and two dispersed none in any form. Only one carrier discharged large numbers, both as "droplet nuclei" and in large droplets, 1.5, 5.5, and 9.5 feet away. About one-half the alpha or beta streptococci sneezed out as droplet nuclei were still present in the air ten to sixteen minutes afterward. This carrier's saliva contained unusually large numbers of hemolytic streptococci.

That saliva is the transport vehicle for material sneezed into the air was demonstrated by the finding of large numbers of salivary (saphrophytic) streptococci in the air as droplet nuclei in 35 per cent of the experiments and in large droplets during 80 per cent.

These observations demonstrate that only rarely does sneezing play an important role in the direct contamination of the air with beta hemolytic streptococci. It may occasionally play a minor part by contributing to secondary bacterial reservoirs in floor dust, handkerchiefs, and elsewhere.

## 89. THE CONTAGIOUSNESS OF COCCIDIOIDOMYCOSIS

SOL ROY ROSENTHAL, M.D., CHICAGO, ILL.

Experiments were conducted in three phases to show that coccidioidomycosis is a contagious disease in guinea pigs:

1. Spherule-containing exudates from patients or infected guinea pigs when instilled into the trachea of guinea pigs produced a primary form of the disease very similar to that found in human beings.

2. (a) Spherule-containing exudates obtained from patients and kept in the icebox for periods up to 110 days failed to produce mycelial threads and chlamydospores but retained their spherule forms that were infective to guinea pigs. (b) Spherule-containing sputum exposed outdoors to the shade or the sun with or without earth for periods up to 230 days retained their spherule forms, albeit in a certain percentage of cases mycelial forms were found.

3. In a limited number of cases, normal guinea pigs placed in the same cages with animals infected intratracheally with the spherules of *coccidioides immitis* became infected.

## 90. THE EFFECT OF GERMAN MEASLES DURING PREGNANCY

STUART ABEL, M.D. (BY INVITATION), AND T. R. VAN DELLEN, M.D.  
CHICAGO, ILL.

A request for letters from mothers who had German measles during pregnancy was included in a syndicated health column. They were asked to state the exact month of gestation that the illness occurred and the effect on the offspring. Over ninety replies were obtained and of these eighty-two were considered acceptable. The series includes two sets of twins, making a total of eighty-four children.

Three stillbirths were recorded from mothers having German measles during the first trimester of pregnancy. Twenty-five of the children were normal at birth. In seven of these the mother contracted the disease during the first trimester, eleven during the second, and seven in the last. Fifty-six of the infants were abnormal at birth, thirty-six with a single defect and twenty with more than one defect. In forty-four (76 per cent) of these the mother told of having German measles during the first trimester of pregnancy, eight in the second, one in the third, and unknown in three. Nineteen had congenital heart disease, seventeen had cataracts, fourteen were deaf, and seven were mentally deficient. Gastrointestinal, eye, spinal, and skeletal abnormalities also occurred in lesser numbers.

The most serious defects or combination of defects occur in women having German measles during the first trimester; less serious and more infrequent during the second. Only one abnormal child was born in the third trimester group. The diagnosis was cerebral palsy and was not considered to be related to the mother's illness.

Statistics obtained reveal that 87 per cent of the babies born of mothers having German measles during the first trimester were abnormal; 42 per cent during the second; and none in the third. These studies confirm previous observations that there is a definite relationship between congenital defects in the child and German measles in the mother.

## 91. ABDOMINAL ACTINOMYCOSIS

GEORGE C. TURNBULL, M.D., EVANSTON, ILL.

The abdominal form of actinomycosis represents a highly fatal form of the disease with very few cases reported in the literature. The clinical course is long and tedious, if recovery occurs. It is not recognized early, as a rule, since it is usually associated with, or follows, other diseases in which there is marked emaciation, abdominal pain, anorexia, chills, fever, sweats and diarrhea or constipation. The infection frequently extends to the liver, spleen, kidneys, and central nervous system. Recovery occurs in approximately 20 per cent of the cases.

One patient is reported in whom an abdominal infection by actinomycosis occurred after removal of the gall bladder because of recurrent gallstone colic. The clinical course was prolonged over a period of nine months with episodes of chills, fever, and the appearance of multiple granulomatous masses in the abdomen. One mass drained spontaneously through the left Fallopian tube and uterus. Recovery occurred after prolonged use of penicillin and sulfadiazine in massive doses, associated with adequate surgical removal of a granulomatous mass.



## 92. COMPARATIVE STUDIES ON THE IODINE ABSORPTION OF ANAYODIN, CHINIOFON, DIODOQUIN, AND VIOFORM IN MAN

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(INTRODUCED BY RALPH C. BROWN, M.D.)

Since direct methods of assay of these oxyquinoline drugs are impractical, their absorption after oral administration\* was studied indirectly by determining blood iodine levels. Two hundred eighty blood iodine determinations were made as follows: before the drugs were started and on the third, the seventh, and the tenth days. No initial level before the drug was taken was higher than 17 gammas of iodine per 100 milliliters. The anayodin average was 94 on the third day, 102 on the seventh day, and 102 on the tenth day. The chiniofon average was 85 gammas on the third day, 87 on the seventh day, and 81 on the tenth day. The vioform average was 297 gammas on the third day, 444 on the seventh day, and 393 on the tenth day. The diodoquin average was 651 gammas on the third day, 692 on the seventh day, and 615 on the tenth day. Consideration was given to diarrhea and other factors possibly influencing absorption. In recommended therapeutic dosage chiniofon and anayodin gave the lowest, vioform the next lowest, and diodoquin the highest absorption levels of iodine. In terms of absorbability as measured by iodine intake and percentage recovery in the blood, vioform is the highest, diodoquin next highest, and anayodin or chiniofon third highest.

The absorption was uniform for each drug and was highest on the seventh day and then declined slightly by the tenth day. The drugs were not cumulative and not toxic. This proved absorption of these drugs raises the question of their effectiveness on amoebae deep in the walls of the intestine or in other parts of the body. It likewise shows that none of them are wholly nonabsorbable. They probably destroy no cysts but act on trophozoites only, thus preventing cyst formation.

## 93. THE EFFECT OF SERUM PROTEIN DEPLETION ON WATER AND SALT EXCRETION AND SENSITIVITY TO PITUITRIN

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CHICAGO, ILL.

Dogs were depleted of protein by a combination of a 4 per cent protein diet and daily plasmapheresis. The effect of serum protein depletion has been investigated with respect to (1) the excretion of water, (2) the excretion of chloride, (3) the extracellular fluid volume, (4) the glomerular filtration rate, and (5) sensitivity to pituitrin.

Plasmapheresis and low protein diets decreased the total plasma proteins from control values of 5.5 to 7.0 Gm. per 100 ml. to 3.5 to 4.5 Gm. per cent; albumin from control values of 3.9 to 2.7 to 1.31 to 1.92 Gm. per cent.

Serum protein depletion had the following effect on the dogs studied: (1) The time required to excrete 50 per cent of ingested water was increased in all dogs following depletion. (2) Protein depletion had no effect on the daily twenty-four hour excretion of chloride. (3) Protein depletion did not appear to alter the twenty-four hour excretion of chloride after the oral administration of 50 c.c. per kilogram of a 0.85 per cent saline solution. (4) During de-

\*Dose given: Vioform, 0.25 Gm. three times daily; all others, 0.75 Gm. three times daily.

pletion the extracellular fluid volume increased slightly in all dogs. (5) The glomerular filtration rate was reduced in all dogs. (6) The urinary suppression produced by pituitrin was increased slightly in all dogs. (7) No gross edema or ascitic fluid was present on autopsy. (8) Serum protein depletion had little effect on the histologic appearance of the liver and kidney except for a loss of cytoplasm in the polygonal cells of the liver.

#### 94. THE DETERMINATION OF THE NITROGEN BALANCE INDEX OF A NEW LYOPHILIZED CASEIN ACID HYDROLYSATE IN PROTEIN-DEFICIENT PATIENTS

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In a study of the clinical acceptability of a new lyophilized casein acid hydrolysate from which some of the glutamic and aspartic acids had been removed (Amino Acids, I.C.),\* nitrogen balances with progressively increasing quantities of the product were carried out in twenty patients with chronic protein deficiency. The material could be injected intravenously in a 10 per cent solution in water, saline, or 5 per cent solution without reactions if the speed was kept below 60 drops per minute. The caloric intake was maintained at about 2,000 calories chiefly with orally administered carbohydrate.

Positive nitrogen balance was obtained in nineteen of the twenty patients studied. The nitrogen intake required to produce nitrogen equilibrium varied from 42 to 340 mg. per kilogram per day, averaging 129 mg. per kilogram, with a standard deviation of 51 per cent. The values were directly proportional to the magnitude of the nitrogen excretion on a protein-free diet, as Allison had found in protein-depleted dogs.

The curve of nitrogen balance in the range of negative and low positive balance was found to be a reasonably good straight line in thirteen experiments. In the remaining six, straight lines could be drawn but were not so well defined. In the region of high positive balance, the curves tended to level off, indicating a ceiling of utilization.

The slope (K) of these lines is the nitrogen balance index of Allison and is a measure of the biologic value of the protein. It averaged 0.68, with a standard deviation of 24 per cent. In the thirteen more precise cases, K averaged 0.66, with a standard deviation of 20 per cent. The nitrogen balance index was therefore a more consistent and more reliable measure of the quality of the protein than the nitrogen intake requirement at equilibrium. The average K value of 0.68 indicated a good protein product. Other experiments show that the biologic value of the protein product was still higher if administered orally.

Serum protein concentrations and total circulating protein were not appreciably increased during the short period of study, results of which confirmed earlier impressions that a much more intense and protracted regimen of protein therapy was required to alleviate severe chronic protein deficiency.

\*Prepared for intravenous injection by the Interchemical Corporation.

## 95. THE EFFECT OF THE RATE OF ADMINISTRATION OF AMINO ACID PREPARATIONS ON THE URINARY WASTAGE OF AMINO ACID NITROGEN

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ANDREW G. LASICHAK, M.D. (BY INVITATION), ELOISE, MICH.

Amino acid mixtures administered intravenously are tolerated at rates exceeding those of casein hydrolysates. This is of practical importance in reducing the time that the patient is kept inactive during intravenous protein alimentation. This study was designed to determine whether the advantages gained by the rapid infusion might be offset by an increased urinary loss. Three different amino acid preparations were studied: Preparation I was an enzymatic hydrolysate of casein (10 per cent Amigen); Preparation II, an acid hydrolysate of casein (Parenamine 15 per cent); and Preparation V (VUJN-9, Merck), a mixture of amino acids prepared by the recombination and fortification of fractions of a casein hydrolysate. The test dose was 500 ml., containing approximately 5.4 Gm. of nitrogen. The amount of amino acid nitrogen in the urine was determined for a four-hour period in twenty-nine individuals.

Five patients received all three preparations at a rate of 500 ml. in two hours; subsequently, as a control, these patients received an equal volume of physiologic saline. The total amino acid nitrogen loss was greater after the administration of Preparation I than after the other preparations. This increase was due mainly to the increased spillage of peptide nitrogen. The amount of amino acid nitrogen in the urine following Preparations II and V were approximately the same. The percentage of the administered amino acid nitrogen in the urine following Preparation I was 10.9 per cent; Preparation II, 5.1 per cent; and Preparation V, 3.9 per cent.

Ten patients received Preparations I and II at rates approaching maximum tolerance, Preparation I being given in one and one-half hours, Preparation II in two hours; Preparation V was given in one hour. Usually less amino acid nitrogen appeared in the urine after Preparation V, in spite of its being given at increased rates.

Four patients received Preparation V, first in a one-hour infusion and later in a thirty-five minute infusion. Three of the four patients had less amino acid nitrogen in the urine following the very rapid infusion than after the one-hour injection; the other lost a little more amino acid nitrogen after the slow infusion.

These studies show that the loss of amino acid nitrogen in the urine does not depend on the rate of the administration of the amino acid preparations. Thus, preparations which are well tolerated may be infused at rapid rates without increasing the urinary spillage of amino acid nitrogen.

## 96. THE EFFECTS OF INFLAMMATION ON THE SEVERITY OF DIABETES

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The concept that gluconeogenesis resulting from proteolysis at the site of inflammation enhanced the severity of diabetes mellitus was introduced by Menkin (1941).

An intense inflammatory reaction was induced by the injection of 1.5 ml. turpentine into the pleural cavity of dogs before and after pancreatectomy. First hourly, then daily, blood and pleural exudate glucose determinations were made. In addition, nonprotein nitrogen, urea nitrogen, and amino acid nitrogen in the blood and pleural exudate were estimated daily. In another experiment glucose was given intravenously and glucose values on blood and pleural exudate were determined hourly. In still other experiments insulin or glucose was injected into the pleural exudate and hourly determinations of glucose in the blood and pleural exudate were done.

We found no sudden marked hyperglycemia following the production of inflammation. While the glucose and nonprotein nitrogen components were higher in the diabetic than in the nondiabetic animals, simultaneous blood determinations showed these values to be higher in the blood of diabetic than in that of nondiabetic dogs.

When Menkin's own data were rearranged to show simultaneous blood and pleural exudate values they confirmed our findings.

Our interpretation of our data, as well as that of Menkin's, does not permit the conclusion that gluconeogenesis occurs locally at the site of a chemically induced inflammation in the depancreatized dog.

## 97. THE ROLE OF HYALURONIDASE IN HUMAN INFERTILITY: PRELIMINARY REPORT

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J. M. BIRKELAND, PH.D. (BY INVITATION), COLUMBUS, OHIO

Since the initial observation that hyaluronidase is found in considerable quantities in the male ejaculate, a relationship between this enzyme and the process of fertilization has been postulated. While observations on human specimens have been made, these have, in general, been confined to men, rather than to the problem of the infertile couple. The present study was undertaken in an effort to determine more exactly the role of hyaluronidase in human sterility.

These investigations have followed four lines: (1) The transportation mechanism to the Fallopian tubes of hyaluronidase deposited in the vagina has been studied by tissue assay. (2) The time factor involved in such transportation has been investigated. (3) The relative fluorescence of human ejaculate and of hyaluronidase preparations of testicular origin has been determined. (4) In infertile couples, the hyaluronidase level of the ejaculate of the men has been compared with control groups, and insemination has been carried out after the vaginal application of hyaluronidase.

### *Methods.—*

1. Tissue assays of the Fallopian tube and the appendix removed at operation were carried out in four cases to determine the base line level. In four

additional cases, 1,000 units of hyaluronidase were placed in the vagina prior to operation and assays of the tubes and appendices performed.

2. Two thousand units of hyaluronidase were introduced intravaginally prior to laparotomy. The Fallopian tubes were excised individually at known time intervals following this application and, together with the appendix, were assayed for hyaluronidase level.

3. The human male ejaculate is markedly fluorescent. The degree of fluorescence was compared with the hyaluronidase level and with dilutions of hyaluronidase extracted from the testes of bulls.

4. A series of ten barren couples was selected in whom all studies of the wife were negative for any factor contributing to the infertility. Forty-four inseminations were carried out employing the husband's semen after preliminary intravaginal introduction of the enzyme. Counts and enzyme titrations on the husband were compared with similar data of ten men of recent parentage.

#### *Results.—*

1. The intravaginal application of hyaluronidase is an effective method of introducing it. The number of units of enzymes per gram of assayed tube was twice that found in the control group. The mechanism, however, must be by blood stream absorption since the appendiceal assays also reveal a sharp increase in the treated group. The appendix is apparently a less efficient absorber of hyaluronidase than the Fallopian tube. (Ratio of 1 to 1.5.)

2. Tubes excised thirty-five minutes after the intravaginal introduction of hyaluronidase show a higher level than tubes excised twenty-five minutes after the enzyme was employed. The time interval required to reach maximum tissue saturation has not been determined.

3. The preparation of hyaluronidase used by us is markedly fluorescent. The fluorescence of prepared hyaluronidase solutions is proportional to the hyaluronidase level. Our series has not been extended sufficiently, as yet, to permit a mathematical correlation between the fluorescence of the ejaculate and the hyaluronidase level. It is possible that fluorometric methods may provide the quickest assay of hyaluronidase levels.

4. The correlations available between the sperm counts, motility, normal forms, and the hyaluronidase levels are discussed. None of the patients, receiving artificial insemination following the administration of hyaluronidase, conceived. It is not felt that this negative finding rules out the possibility of hyaluronidase playing a role in the mechanism of fertility. It does not appear, however, that a deficiency of hyaluronidase represents the explanation for this series of unexplained infertile couples.

### 98. SERUM PHOSPHATASE IN OSTEOPETROSIS

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In the 138 cases of osteopetrosis (Albers-Schönberg's disease; marble bones) reported in the literature, no record has been found of the acid phosphatase level, and very few specific references to normal basic phosphatase are noted.

A case of osteopetrosis who also showed evidence of two other congenital osteopathies (osteopoikilosis and melorheostosis), occurring in a man of 57 years, has been studied in detail, clinically, roentgenologically, and in the laboratory. For nearly two years repeated determinations have been made of serum alkaline and acid phosphatase. The basic phosphatase has always been in the normal range. The acid phosphatase has, with a single exception, been

consistently elevated. On four occasions figures of 10, 10.8, 12.1, and 12.3 King-Armstrong units were obtained—a level considered as diagnostic for carcinoma of the prostate which has broken through the capsule of the gland. However, serial sections of prostatic tissue removed by transurethral resection showed no evidence of malignancy in this patient.

Only one instance has been previously reported in which a significant elevation of blood acid phosphatase has occurred unassociated with carcinoma spreading from the prostate. This was in an 18-year-old girl with hyperparathyroidism who had a greatly increased basic phosphatase at the time. It is granted that radiographic evidence of osteoblastic metastatic lesions from carcinoma of the prostate might well be concealed by the widespread condensing or sclerosing osteopathy found in osteopetrosis. However, the likelihood of one individual having two conditions, one moderately uncommon and the other extremely rare, does not seem great.

The purpose of this communication is to urge that whenever a case of this very rare congenital bone disorder, osteopetrosis, is encountered, observations of serum acid phosphatase be made in an effort to evaluate the findings in this one patient.

## 99. CEREBROSPINAL FLUID STUDIES IN ARTHRITIS

MAURICE HARDGROVE, M.D., JOSEPH FURLONG, M.D. (BY INVITATION),  
GEORGE MURPHY, M.D. (BY INVITATION), MILWAUKEE, WIS.

Cerebrospinal fluid examinations were made in twenty-one patients having various types of arthritis.

There were ten patients in the group who had rheumatoid arthritis; one of these also had rheumatoid spondylitis. Although the ages in this group varied from 23 to 60 years, all of the patients had active lesions and elevated sedimentation rates. The duration of the illness was from two months to twenty years, and a variety of joint deformities were manifest. In one there was a mild disturbance in the colloidal gold curve. This was the most acute and severe case seen during the study. Three of the patients had spinal fluid proteins above 30 but none were above 40 mg. per 100 milliliters. One fluid showed a slight increase in globulin. The patient who had rheumatoid spondylitis in this group showed normal spinal fluid findings.

In five additional patients having rheumatoid spondylitis there were no colloidal gold curve changes; one showed a slight increase in globulin.

No abnormal gold curves were found in four patients with severe osteoarthritis of the spine. In one of these the total protein was elevated to 50, though the Queckenstedt was normal.

One patient with fibrositis (muscular rheumatism) with an elevated sedimentation rate showed no spinal fluid changes.

One patient was considered to have psychogenic rheumatism and showed no spinal fluid abnormalities.

The blood serology and the spinal fluid Wassermann reactions were all negative. The fluid was clear and sterile in all cases and in no case was the cell count above 5.

The spinal fluid findings have not been instructive in this small group of patients.

## 100. CYTOLOGIC STUDIES OF SPUTUM IN BRONCHOGENIC CARCINOMA

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Since April, 1946, various body fluids have been subjected to cytologic study by the method of Papanicolaou.

Sputum from ninety patients with pulmonary lesions has been examined. A brief outline of the methods employed in preparation of specimens is presented.

Positive findings of carcinoma were reported in twenty-three cases. In seven cases confirmation was obtained at autopsy or exploration. Bronchial adenoma rather than carcinoma was found at exploration in one instance and tuberculosis in another. In eleven cases presumptive confirmation was obtained from roentgenographic findings, clinical course, and death. Subsequent course is unknown in three cases. In four cases the sputum was considered suspicious and on further study it was concluded that squamous metaplasia of the bronchial epithelium had resulted from an irritative focus overlying a calcified bronchial node or from bronchiectasis.

Certain diagnostic pitfalls are discussed.

It is concluded that cytologic examination of the sputum when carefully carried out by an experienced observer can be a valuable aid in the diagnosis of bronchogenic carcinoma and can give positive results when bronchoscopy has failed and other studies are inconclusive.

## 101. CHRONIC BILATERAL BASAL PULMONARY FIBROSIS: (CLINICAL STUDY)

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DETROIT, MICH.

A study has been conducted of the etiology and course of basal pulmonary fibrosis as revealed by x-ray in ninety-nine patients. Special examinations included chest x-ray, electrocardiogram, sputum studies, and vital capacity. When advisable, bronchograms, bronchoscopies, allergy studies, venous pressures, and circulation times were obtained. Patients with sinus or oral infections were excluded. Diagnostic effects of antibiotics were utilized. In suspected cases, treatment for congestive heart failure or allergy was helpful in diagnosis.

The studies revealed that the x-ray finding of bilateral basal fibrosis was associated with specific conditions in eighty-one patients. The causative factors were congestive heart failure, bronchiectasis, basal pneumonitis, pulmonary artery sclerosis, lymphogenous dissemination of gastric carcinoma, infiltrative bronchogenic malignancy, inhalation diseases, cystic fibrosis of the pancreas in children, pulmonary emphysema, bronchial asthma, and fibrosis secondary to x-ray therapy. Fungus disease was not discovered. No case resulted from continued use of a tracheal airway. Also none was related to chronic nephritis, idiocy, mental deterioration, or prolonged unconsciousness. Abdominal compression was associated with basal lung densities. More diffuse and less dense infiltration occurred infrequently in diabetic patients.

No specific etiology was established in eighteen patients (18 per cent). The period of observation extended from six months to ten years. These patients had cough, basal râles, and bilateral basal pulmonary fibrosis. Eleven

were women and seven were men. The average age was 56 years, with extremes of 43 and 73 years. Handicapping dyspnea was present in eleven. Fever was known to be present at the onset in three. It occurred intermittently in three others. Four electrocardiograms showed minor abnormalities. Left bundle branch block occurred once. In one case 16 per cent eosinophilia occurred during a period of fever. Reproduced chest x-rays revealed different degrees of fibrosis. In two instances improvement in the x-ray findings was noted. In one of these, lung puncture was done six years ago.

This subject is presented in the belief that increased and improved diagnostic efforts will result in the discovery of specific etiologies. Lung puncture may be justified. Possibly pulmonary artery sclerosis is the principal unrecognized factor. Some cases represent chronic recurrent infections. Exogenous nutritional deficiency was not suggested by these studies. Thyroid, pancreatic, or other endocrine abnormalities may prove to be of importance. Disturbed tissue metabolism with exudation and proliferation in excess of absorption and drainage may be a factor. The one consistent feature is age with its increased tissue vulnerability.

## 102. FEVER THERAPY IN HERPES ZOSTER

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True herpes zoster is generally considered as caused by a varicella-like virus involving one or more posterior root ganglia resulting in pain and inflammation of the corresponding dermatomes. However, any of several diseases which involve the dorsal ganglia in an inflammatory process may give rise to "shingles" and it is important to differentiate this symptomatic group. Other nerves, notably the trigeminal, may be affected.

The typical evolution of the lesion and its specific characteristics are well known, and the therapy of the condition has been polypragmatic, with varying and sometimes indifferent results.

The nature of the causative agent as well as the site of the lesion suggested fever therapy. This was produced by mixed typhoid vaccine injected intravenously. A temperature of 102 to 106° F. was sought, preferably the latter. Usually this was preceded by a shaking chill.

Six of eight patients with true herpes zoster have been so treated, with striking benefit. If treated early, pain disappears promptly and the skin lesions quickly involute. No postherpetic pain has been encountered in the group. The lesions treated were primarily on the chest or the abdomen, although one involved the supraorbital nerve and another the superior cervical.

The selection of cases is important. Elderly individuals, or those with symptomatic herpes, should not be so treated. Late or well-established cases are likely to have a less favorable outcome. There have been no untoward results and postfebrile herpes labialis has been the only complication. Vitamin B<sub>1</sub> has had little effect in modifying or preventing this. However, the slight discomfort entailed is more than offset by the prompt relief of the neuritic pain. Artificially induced fever appears to have a definite place in the therapeutic armamentarium of true herpes zoster.



### 103. CLINICAL OBSERVATIONS ON THE USE OF THEPHORIN (NU-1504): A NEW ANTIHISTAMINIC AGENT

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Thephorin is a new antihistaminic agent, originally known as NU-1504, which has been developed in the Roche Research Laboratories.

It is a polycyclic amine, the empiric formula of which is  $C_{19}H_{19}N$  (2-methyl-9-phenyl-tetrahydro-1-pyridindene). It belongs to a heretofore unknown class of compounds. The tartrate which we have used in this study is soluble in water and in a 2 per cent aqueous solution; its  $pH = 5$ .

Experimental studies in animals demonstrate the antagonism to histamine by (1) abolition of histamine-induced contraction in the isolated guinea pig intestine; (2) relaxation of spasm resulting from treatment with acetylcholine or barium in the rabbit intestine; (3) prevention of broncheolar contraction and convulsions in guinea pigs exposed to histamine spray; (4) prevention of the hypotensive effect of histamine given intravenously in cats; and (5) reduction in size of wheals produced by intradermal injection of rabbits or human subjects. Studies for toxicity show that the dosage of thephorin which was fatal to 50 per cent of animals compares almost exactly with that of benadryl but that thephorin is less toxic than pyribenzamine. There is no evidence of chronic toxicity in animals.

The purpose of our study has been to investigate the use of this agent in subjects with symptoms thought to be due in whole or in part to the release of H substance. To date, we have treated fifty-two patients with thephorin. Of this group, thirty-two showed evidence of definite improvement and twenty showed no improvement.

In eighteen patients with hay fever, fourteen reported excellent results on a dosage of 100 mg. daily. Of these, only twelve were relieved by benadryl but had to discontinue its use because of a hypnotic effect. Thirteen were relieved by pyribenzamine but stopped taking it because of gastrointestinal symptoms. Four of our patients did not have their symptoms adequately controlled by thephorin, although the dosage was 200 mg. daily. Three of the four subjects also failed to obtain relief with benadryl and pyribenzamine. Nine patients with vasomotor rhinitis were treated, and four had excellent results with clearing of the rhinitis and sinusitis. The remaining five had no improvement. Of six patients with chronic urticaria, one patient showed improvement and five showed no change. In four patients whose urticaria was on the basis of a physical allergy, two experienced excellent results, and in the other two no evaluation could be made. Six patients with histaminic cephalgia were treated. Two of them had definite improvement. Five patients with migraine and nervous tension headaches were given thephorin; only one of them reported good results. One patient with Ménière's disease was not benefited. One patient with Raynaud's disease, one with erythromelalgia of the hands, and one with dermatographia showed improvement.

No serious toxic symptoms have resulted from the use of this drug. One patient complained of drowsiness. In two patients treated over a period of ten months, the results of routine laboratory studies remained unchanged.

This study indicates that thephorin is a useful drug for the treatment of edema problems in which the etiologic factor is probably the release of H substance. Our comparison of this agent with benadryl has demonstrated that some

patients will respond to thephorin when they do not respond to benadryl, that the dosage required for the control of symptoms was at least 50 per cent less, and that there is an absence of unusual toxic symptoms.

#### 104. THE TREATMENT OF MIGRAINE WITH HISTAMINE: REVIEW OF 144 CASES

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One hundred forty-four patients with migraine were treated with histamine therapy at the Mayo Clinic between 1937 and 1945. These patients have been divided into two groups, one of which includes 124 patients treated prior to June, 1945, and the other, twenty patients treated during the period from June through September, 1945. The cases in each series were classified as typical or atypical migraine. Histamine was administered intravenously, subcutaneously, or by both routes. It was not supplemented by any other type of therapy.

Statistical analysis of data on these cases has shown that the migraine syndrome of 23 to 33 per cent of patients was unchanged by histamine therapy. The syndrome of 33 to 50 per cent of patients with typical migraine, and that of 40 to 66 per cent of patients with atypical migraine, showed significant improvement during treatment by the intravenous route. Approximately 60 per cent of migrainous patients, irrespective of the type of migraine, showed significant improvement during the period of subcutaneous administration of histamine. When histamine was administered by both routes, 70 to 85 per cent of patients with typical migraine and 75 to 100 per cent of patients with atypical migraine showed significant improvement during treatment.

Of the eighty-eight patients who exhibited improvement during the period of treatment, and on whom follow-up data were complete, eighty-five experienced a recurrence of the migrainous attacks when the dose of histamine was decreased below an individual critical level. The three patients who had not experienced a recurrence of their attacks of migraine were still taking histamine subcutaneously at the time they reported to us. A tendency was noted for typical migraine to be more refractory to treatment with histamine and to recur sooner after such treatment than atypical migraine.

There appeared to be no constant relationship between either the total dosage or the maximal single dosage of histamine base and the degree or duration of abatement of the migraine syndrome. It was found that migrainous patients, in general, tolerate ten times more histamine base in a single amount administered by the intravenous route than by the subcutaneous route. In migrainous patients there appeared to be a qualitative as well as a quantitative difference between the effects of histamine administered intravenously and when it was given subcutaneously.

The use of histamine may prevent some attacks of migraine in certain cases. The duration of such an effect seems to parallel the duration of administration of adequate amounts of the drug. Histamine does not appear to be specific in the treatment of migraine.

# 105. MASKED FOOD ALLERGY AS A FACTOR IN THE DEVELOPMENT AND PERSISTENCE OF OBESITY

Theron G. Randolph, M.D., Chicago, Ill.

The voracious appetite of the patient who apparently becomes obese as a result of eating an excessive number of times per day has been explained variously as due to habit, the gratification of eating, or as a result of "nervous" influences. Another mechanism, heretofore not considered in respect to obesity, may explain this apparent craving to eat frequently observed in certain patients with chronic food allergy.

If an allergenic food is eaten several times daily, a masked or chronic smoldering allergic reaction may develop which is characterized by an improvement in chronic symptoms occurring immediately after eating a specific food allergen and which persists for approximately two hours but is then followed by a progressive increase in symptoms. These patients learn to avoid sharp reactions by eating the foods to which they are allergic at such frequent intervals as for instance at 10:30 A.M., and 3:00, 5:00, and 10:00 P.M. and occasionally during the night, in addition to their three regular meals. Without such interval feedings these patients are inclined to develop midway between their regular meals any one or several of the following symptoms: a gnawing hunger sensation in the abdomen, nasal stuffiness, inability to concentrate, somnolence, extreme fatigue, tenseness, and "nervousness."

It is of interest that the foods most frequently eaten in between meals are those to which there is the highest incidence of sensitivity; namely, corn, wheat, and milk, all of which, incidentally, are also high in calories.

It is exceedingly difficult for these patients with uncontrolled masked food allergy to adhere to a reduction diet; the majority of obese patients seen have previously failed to do this.

Hoetzel's theory, that excessive appetite is associated with tissue hydration, finds suggestive confirmation in these individuals, for the avoidance of masked food allergens is often followed by a diuresis, the disappearance of clinical evidences of edema, and a sudden decrease in weight, all of which are associated with the cessation of the abnormal appetite and an improved ability to follow through on a reduction diet.

# 106. THE INCIDENCE OF ALLERGY TO MAJOR FOODS

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Chicago, Ill.

Two hundred consecutive cases suspected of food allergy and studied by means of individual food tests with corn, wheat, milk, and eggs were selected from the private practice of one of the authors and subjected to analysis. Each patient was fed a single test food under fasting and basal conditions after a four-day preparatory period during which the food to be tested was completely avoided. The incidence and severity of all symptoms were recorded per unit of time prior to and after experimental feeding, each patient remaining under observation for two hours. Total leucocyte determinations were performed prior to and at twenty-minute intervals following the experimental feeding of the first of two doses which were given an hour apart.

The presence of an unmistakable symptom response in association with the food test represented the prime criterion for diagnosis; the presence of a leuco-

penia of 10 per cent or greater occurring in any of the postingestive determinations was regarded as presumptive evidence of sensitivity. Each instance of leucopenia occurring in the absence of symptoms was checked by the cumulative ingestion of the food in question.

In the first 100 cases corn sensitivity was diagnosed by feeding canned corn or corn meal gruel. In the second group of 100 cases corn meal gruel plus corn sugar was used as the test food. The results of these two series indicate that the addition of corn sugar increases the incidence and severity of allergic symptoms occurring during the course of experimental food tests for detection of corn sensitivity.

The data indicate that corn sensitivity ranks in incidence with that of wheat and is followed closely by milk and eggs in the order named allergy to corn being present in eighty-seven cases, wheat in eighty-five, milk in seventy-five, and eggs in sixty-seven.

Patients cannot be depended upon to detect sensitivity to a major food allergen. In this series a major food was suspected correctly of causing current allergic symptoms in only 8.0 per cent of the cases.

The high incidence of corn sensitivity shown by these data make it imperative that corn allergy be considered in all cases suspected of food sensitivity. It is significant that practically all diagnostic elimination diets widely employed in the past have not completely eliminated corn as an allergen.

## 107. GELATIN AS AN ALLERGEN

Theron G. Randolph, M.D., CHICAGO, ILL.

In view of the reported nonantigenicity of ossein gelatin and the fact that beef gelatin solutions are commonly used intravenously as plasma substitutes, it is of interest to know that even highly refined ossein gelatin preparations may produce symptoms when ingested by beef sensitive patients.

Four patients with beef sensitivity, each diagnosed by the production of acute allergic symptoms during individual food tests with beef, were subjected to individual food tests with commercial Gelatine<sup>a</sup> and Gelatine<sup>c</sup> employed for intravenous use.

The experimental ingestion of Gelatine (ossein) dissolved in water and taken fasting in two doses of  $\frac{1}{4}$  ounce each an hour apart was associated with the following reactions:

### Controls

Four patients known not to be beef sensitive—no reaction

### Beef-Sensitive Patients

Case 1.—No reaction

Case 2.—Nasal stuffiness, chilliness, dizziness, abdominal distention, and cramps

Case 3.—Fatigue, headache, tinnitus, abdominal distention, nausea, vomiting and diarrhea—the latter persisting two days

Case 3.—Violent paroxysms of sneezing, followed by nausea and severe headache

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<sup>a</sup>Supplied through the courtesy of the Chas. B. Knox Gelatine Co.

The experimental ingestion of 150 ml. and one hour later 75 ml. of Gelatine of beef origin prepared for intravenous use was associated with the following reactions:

Case 1.—No reaction

Case 2.—Nasal stuffiness, chilliness, scotomas, somnolence, and abdominal distress

Case 3.—Chilliness, muscle aching, marked fatigue, indigestion, headache, and delayed peripheral edema

Case 4.—Not subjected to this test because of the violent nature of reaction to beef and to commercial gelatin.

It is of interest that the beef sensitivity in each of these cases was completely masked and was not suspected by any patient in spite of the high degree of sensitivity which existed. Inquires of undiagnosed patients as to whether or not they are known to be beef sensitive are usually worthless.

Pork sensitivity did not exist in any of the four patients studied as determined by compatible individual food tests with pork (complete absence of symptoms and a trajectory curve of leucocytosis.) In each instance commercial porcine Gelatine was tolerated in individual food testing without symptoms.

## 108. THE ELECTROENCEPHALOGRAM IN TUMORS OF THE POSTERIOR FOSSA

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(INTRODUCED BY GRACE M. ROTH, PH.D.)

The electroencephalographic findings in a group of fifty predominantly adult persons who had tumors of the posterior fossa can be classified as follows: (1) normal records in 6 per cent of cases; (2) paroxysmal rhythms with frequencies of 3 to 7 and 18 to 22 cycles per second, with a tendency to repetitive wave forms and synchronous activity from all parts of the head in 56 per cent, and (3) a less uniform group showing continuous delta rhythms with a frequency of 2 to 6 cycles per second and delta foci unrelated to the site of the tumor in 38 per cent of cases.

Many of the characteristic abnormalities were seen to advantage in transverse leads from homologous parts of the scalp. For instance, in 65 per cent of all records abnormal waves were noted in the interear lead.

The alpha rhythm showed a marked instability of frequency (in excess of 2 cycles per second) in 42 per cent of the whole group, whereas in another 14 per cent the alpha rhythm was completely disorganized by delta waves. A fast activity with a frequency between 18 and 22 cycles per second, either continuous, or in episodes, was present in 20 per cent of records. A further characteristic feature was the inhibition of abnormal activity when the patients opened their eyes. This occurred in 60 per cent of cases.

The group of records which showed paroxysmal rhythms is interesting since the majority of tracings were indistinguishable from those seen in cases of epilepsy; even the classical wave forms were reproduced. These rhythms, however, cannot represent true epileptic activity since clinical epilepsy from these tumors is almost unknown.

The resemblance might be explained on the basis of a common diencephalic origin for the rhythms of epilepsy and those associated with tumors of the posterior fossa. In the latter case, expansion of the third ventricle which is known to be an early event in the progress of tumors of the posterior fossa presumably would initiate the diencephalic disturbance.

From the diagnostic point of view, it is suggested that the occurrence of a rhythm typical of epilepsy in the tracing from a patient without previous or family history of epilepsy should arouse a suspicion of tumor of the posterior fossa.

## 109. PERNIO: AN INSTANCE OF APPARENT RECOVERY

DOUGLAS DEEDS, M.D., M.S., DENVER, COLO.

This rare case is being reported because of several interesting features. In 1938 the patient, a white woman, 36 years of age, experienced following exposure to cold the onset of recurrent attacks of painful, disfiguring skin lesions on the ankles and lower extremities only. From that time until seen by the author in 1942 a variety of utterly ineffective therapeutic regimes had been tried. The author advanced the diagnosis of pernio in April, 1942. Subsequent corroboration by Dr. Irving S. Wright and by the vascular section of the Mayo Clinic was greatly appreciated, and the assistance so generously offered by these two sources was of inestimable value.

Otherwise the patient was very healthy. Repeated and exhaustive studies failed to reveal any other pathology. Many ineffectual therapeutic agents had been tried. The author urged sympathectomy and used intravenous typhoid vaccine temporarily since it hastened regression of the unsightly blemishes. No permanent effect of any sort was noted following this form of therapy.

In April, 1943, bilateral sympathectomy was performed. Satisfactory interruption of the sympathetic pathways to the lower limbs was proved by post-operative sweating, etc., tests. Despite this the results were unsatisfactory for the recurrent lesions continued to appear upon exposure to cold. The only apparent effect of sympathectomy on the pernio lesions was to accentuate the severity of the pain associated with the lesions. Kodachrome slides demonstrate the lesions before and after sympathectomy.

In an attempt to assist the patient with her distressing problem another theoretical approach was followed. Fractional gastric analyses revealed hypochlorhydria. Dilute hydrochloric acid administered orally thrice daily during meals controlled long-standing, mild, gaseous indigestion. The patient felt that there was some beneficial effect on the ease of precipitation and severity of the pernio lesions.

Histamine desensitization was then undertaken. The initial dose was 0.05 mg. of actual histamine base subcutaneously thrice weekly with gradual increase over a two-month period to 0.35 milligrams. Excessive reaction then necessitated reduction in dosage. A maintenance ration of 0.2 mg. thrice weekly was maintained until the end of cold weather. Despite continued residence at a mile high altitude and repeated and often intentional exposures to cold, no new lesions appeared.

The author wishes to emphasize the need for further investigation of pernio and that sympathectomy was utterly ineffective in this case. An apparently successful therapeutic program is described.

## 110. CIRCULATORY RESPONSES TO SPINAL AND CAUDAL ANESTHESIA IN HYPERTENSION: RELATION TO THE EFFECT OF SYMPATHECTOMY

### I. EFFECT ON ARTERIAL PRESSURE

ROBERT D. TAYLOR, M.D., ROBERT BIRCHALL, M.D., A. C. CORCORAN, M.D.,  
AND IRVINE H. PAGE, M.D., CLEVELAND, OHIO

In 1944 we reported before this Society the effects of high spinal anesthesia on arterial pressure and renal circulation in patients suffering from essential hypertension. In most of them induction of anesthesia resulted in a decrease in arterial pressure. On the assumption that the effects on the circulation of high spinal anesthesia were, in large measure, similar to those resulting from sympathectomy (lumbodorsal sympathectomy and ganglionectomy), we suggested that the response of arterial pressure to high spinal anesthesia might aid in selecting patients for this operation. Caudal anesthesia differs from spinal anesthesia in that it does not paralyze the nerve supply of voluntary muscle. Since it paralyzes preganglionic sympathetic fibers, the denervation is very similar to that of sympathectomy. Such considerations led Russek, Southworth, and Zohman to the conclusion that high caudal anesthesia caused reduction in blood pressure in many patients suffering from hypertension which paralleled the effect on arterial pressure of subsequent surgical sympathectomy. They recommended measurement of arterial pressure during high caudal anesthesia as a guide to selection of patients for sympathectomy.

The purpose of this report is to examine the relationship between the effect on arterial pressure of high spinal or caudal anesthesia and the effect of subsequent sympathectomy by the technique of Smithwick.

The effects of caudal and spinal anesthesia on arterial pressure in hypertensive patients are similar. Among forty-three patients, marked decrease in pressure was observed during anesthesia in forty. Blood pressure was persistently decreased by lumbodorsal sympathectomy and ganglionectomy in twelve of these patients. The three patients whose arterial pressures were not decreased by anesthesia were similarly unaffected by operation.

It is concluded that the blood pressure response to spinal and caudal anesthesia has no more than negative value in the selection of patients for sympathectomy. The discrepancy between the effects on arterial pressure of spinal or caudal anesthesia and sympathectomy may be due to the great difference in time during which the effects are observed or to a difference in the type of denervation which each of them causes.

## 111. PROTEIN METABOLISM IN RHEUMATOID ARTHRITIS

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Nitrogen balance studies were carried out on eight ambulatory and afebrile patients with rheumatoid arthritis and on two normal subjects. Attention was centered on the degree of nitrogen retention following change from a low protein ( $\frac{3}{4}$  Gm. per kilogram of body weight) to a high protein intake (2 Gm. per kilogram of body weight). Diets were constant for successive three-day periods and observations were made for at least three periods at each intake level. Caloric intake was constant throughout and permitted maintenance or increase in weight in each subject.

At the lower intake level all subjects were in slightly negative or neutral nitrogen balance. The normal subjects showed definite nitrogen retention during the first few days after change to the high protein intake. The response of the patients with rheumatoid arthritis to the increase in dietary protein varied and may be classified in three groups as compared with the controls. In four of the eight, the degree of nitrogen retention did not differ significantly from that of the normal subjects. In two arthritic patients, the magnitude of nitrogen retention and its duration appeared greater than in the controls. The remaining two arthritic patients retained very little nitrogen during the first few days of high protein feeding. The pattern of the nitrogen retention did not appear related to the severity or duration of the arthritis. Greater than normal retention occurred in the patients who were most markedly underweight.

Nitrogen balance was followed on one additional underweight patient with rheumatoid arthritis during a three-month period on a constant diet supplying a liberal caloric and protein intake. The degree of nitrogen retention was not in excess of that expected to accompany the weight gain which occurred.



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